

CHEMISTRY and PHYSIOLOGY of the Vitamins

By

H R ROSENBERG, Sc D

Revised Reprint

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PREFACE TO THE REVISED REPRINT

The present volume is essentially a reprint of the 'Chemistry and Physiology of the Vitamins' which was published in 1942. Although many new discoveries have been made since the manuscript for the first edition was written, practically none of these has been incorporated into the reprint. Only actual errors which have crept into the first edition and which have come to the attention of the author have been corrected. However, the Recommended Daily Allowances, as published in January 1943 by the Food and Nutrition Board of the National Research Council, have been added.

Wilmington, Del
Spring 1945

H. R. ROSENBERG

PREFACE TO THE 1942 EDITION

This monograph *Chemistry and Physiology of the Vitamins*, has a long historical background. Exactly a decade ago it was the privilege of the author to be present at the meeting held by the Zurich section of the Swiss Chemical Society when Paul Karrer announced a few days prior to its publication the successful isolation of essentially pure vitamin A from fish liver oils and the establishment of the structure of this vitamin. It was then that the author felt the urge to compile the available data on the chemistry and physiology of all vitamins. The science of vitamins had by that time advanced to a state at which the existence of vitamins, brought into the limelight between 1906 and 1914, was no longer questioned. In 1926 B. C. P. Jansen and W. F. Donath had announced the isolation of pure, crystalline vitamin B₁, but the constitution of this vitamin remained unknown for many years to come. At the same time (1926), *Pohl, Windaus, Hess, Rosenheim and Webster* had recognized that the long known ergosterol could be converted into a vitamin D by activation with ultraviolet light, but the pure vitamin D was not obtained until 1931-1932, and the correct chemical constitution was not known. Thus, Karrer was the first to establish the true chemical constitution of a vitamin.

In the years following Karrer's announcement, the author, while working in the laboratories of L. Ruzicka and T. Reichstein, had the good fortune of being able to see and observe the progress made on the establishment of the structure and the synthesis of several vitamins and hormones. During that time Reichstein synthesized vitamin C, the first vitamin ever obtained by total synthesis. And, Karrer, in a laboratory only a few blocks removed, worked in a dramatic race against Kuhn in Germany on vitamin B₁₂, and was the first to announce the successful synthesis of this vitamin. From Ruzicka's laboratory the synthesis of the male sex hormones androsterone and testosterone, by degradation of cholesterol was announced, and Reichstein started to investigate the hormones of the adrenal cortex.

The author was then tempted to plan the publication of a comprehensive volume on the chemistry and physiology of the vitamins and hormones. These two classes of compounds have so much in common that a review of one of them seemed to necessitate a review of the other. The extraordinary activity of research workers in all parts of the world, however, has resulted in the accumulation of such an enormous amount of scientific and practical material that it became infeasible to combine a discussion of

both the vitamins and the hormones in one volume. Thus, this monograph is confined to a treatment of the vitamins exclusively.

Since the author left the hospitable Swiss Laboratories, he has been connected, at some time or other, with the development of many of the vitamins known today. Simultaneously, the need for an up to date presentation of the chemistry and physiology of the vitamins became more and more recognized, and this need is felt today by everyone who desires to inform himself or others on this subject. Short tabulations of the vitamins and scattered review articles do not answer the need. Books written for the general public are obviously of a different category than books of a purely technical character. Fortunately, there have been available in the English language a number of excellent books on the medical aspects of vitamin therapy such as 'The Vitamins,' a symposium published under the auspices of the American Medical Association and 'The Avitaminoses' by W. H. Eddy and G. Dalldorf. There is also a very satisfactory book on 'The Biological Standardization of the Vitamins' written by K. H. Coward. In addition there are monographs on special vitamins such as the book on Vitamin B₁ by R. R. Williams and I. D. Spies, Vitamin D by C. I. Reed, H. C. Struck and I. E. Steck, Vitamin K by H. R. Butt and A. M. Snell and Vitamin E a symposium held under the auspices of The Food Group of the Society of Chemical Industry. The author, however, knows of no comprehensive treatment of the chemistry and physiology of all the vitamins.

The present monograph on the Chemistry and Physiology of the Vitamins begins with the presentation of a definition of the vitamins which distinguishes this group of compounds sharply from the hormones and from other essential and non essential food constituents. A new classification for compounds which have the dual character of vitamins and structural building units or suppliers of energy is introduced. Each vitamin is then discussed separately, emphasis being laid upon the chemistry and the physiological action of these compounds. The chapters on each vitamin start with a review of the nomenclature and a tabulation of the historical development followed by a paragraph on the occurrence of the vitamin. The main discussions on the chemistry and physiology follow. Under the chemistry of each vitamin the procedures used for the isolation of the vitamin, the proof of the chemical constitution and the synthesis of the vitamin are reviewed separately. There are special paragraphs on industrial methods of preparing the vitamins and on their biogenesis. The specificity of the vitamin action is treated separately. The determination of the vitamins is subdivided into physical, chemical

biochemical and biological methods, and is followed by a paragraph on the vitamin standards. The physiology of plants and microorganisms is separated from the animal physiology which is subdivided into the metabolism of the vitamin, the physiological action and the mechanism of the vitamin action. The relation of each vitamin to other vitamins to hormones, and to inorganics is presented in special paragraphs. This is followed by a short review of the present day knowledge of the pathological aspects, the hypovitaminoses, avitaminoses, hypervitaminoses and paravitaminoses, with a special section on clinical test methods. Finally the vitamin requirements are briefly stated. The book ends with a list and abstracts of vitamin patents which have issued in the United States of America, Great Britain, Germany and France arranged in a manner similar to that of the main text.

The vitamins are presented according to the alphabetical order of nomenclature. This arrangement follows in general the incidental discovery of the vitamins. Eventually, a classification according to the function of the vitamins will probably prove to be more satisfactory, but cannot successfully be undertaken at this time due to the rather incomplete knowledge of the primary function of many members.

This volume has been prepared with the idea of covering all topics of vitamin research and especially the chemistry and physiology of the vitamins. In presenting this monograph to the public the hope is expressed that it may guide the student and the scholar through our present day knowledge of the field and inspire further development. This is especially desirable and should be expedited as much as possible since the health and successful propagation of man are, to a considerable extent, dependent upon proper nutrition, and any advance in the knowledge of the vitamins can be utilized immediately for the benefit of mankind.

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TABLE OF CONTENTS

CHAPTER	PAGE
Preface to the Revised Reprint	v
Preface to the 1942 Edition	vi
The Vitamins in General	3
1 Definition of Vitamins	3
2 History of the Discovery of the Vitamins	7
3 Nomenclature	12
4 List of the Vitamins	14
5 Occurrence	16
6 Isolation	17
7 Chemical Constitution	17
8 Synthesis	17
9 Industrial Methods of Preparation	18
10 Biogenesis	19
11 Specificity	19
12 Determination	21
13 Standards	24
PHYSIOLOGY	24
14 Physiology of Plants and Microorganisms	26
15 Animal Physiology	26
(a) Metabolism of the Vitamins	26
(b) Physiological Action of the Vitamins	27
(c) Mechanism of the Vitamin Action	28
(d) Relation of the Vitamins to Each Other to Hormones and Minerals	29
PATHOLOGY	31
16 Avitaminosis and Hypovitaminosis	31
(a) Clinical Test Methods	32
17 Hypervitaminosis	32
18 Requirements	33
The Group of Vitamins A	37
1 Chronology	37
2 The Group of Vitamins A	37
PROVITAMINS A	38
3 Occurrence	38
4 Properties	39
5 Isolation	43
6 Chemical Constitution	46
* 7 Synthesis	49
8 Industrial Methods of Preparation	49
9 Biogenesis	50
10 Determination	51
CONVERSION OF PROVITAMINS A INTO VITAMINS A	53

CHAPTER	PAGE
VITAMIN A	57
11 Nomenclature and Survey	57
12 Occurrence	58
13 Properties	60
14 Isolation	60
15 Chemical Constitution	61
16 Synthesis	65
17 Industrial Methods of Preparation	66
VITAMIN A ₂	68
OTHER VITAMIN A FACTORS—VITAMIN A ₃	71
VITAMINS A	73
18 Biogenesis	73
19 Specificity of the Vitamin A Action	74
20 Determination	78
(a) Chemical Methods	78
(b) Physical Methods	80
(c) Biological Methods	82
21 Standards	82
22 Physiology of Plants and Microorganisms	83
23 Animal Physiology	84
(a) Metabolism of Provitamins A and of Vitamins A	84
(b) Physiological Action of Provitamins A and of Vitamins A	87
(c) The Visual Purple	89
(d) Relation of Vitamins A to Other Vitamins Hormones etc	90
24 Avitaminosis and Hypovitaminosis	91
(a) Clinical Test Methods	93
25 Hypervitaminosis	95
26 Vitamin A Requirements	95
Vitamin B ₁ —Thiamin	99
1 Nomenclature and Survey	99
2 Chronology	100
3 Occurrence	100
4 Isolation	101
5 Properties	103
6 Chemical Constitution and Synthesis	104
(a) The Pyrimidine Part	105
(b) The Thiazole Part	110
(c) The Connection of the Pyrimidine and the Thiazole Part	113
7 Industrial Methods of Preparation	118
8 Biogenesis	119
9 Thiochrome	120
10 Vitamin B ₁ Pyrophosphate	122
11 Specificity of the Vitamin B ₁ Action	126

CHAPTER	PAGE
12 Determination	128
(a) Chemical Methods	128
(b) Biological Methods	130
(c) Biochemical Methods	131
13 Standards	132
14 Physiology of Plants and Microorganisms	132
15 Animal Physiology	134
(a) Metabolism of Vitamin B ₁	134
(b) Physiological Action of Vitamin B ₁	135
(c) Mechanism of the Vitamin B ₁ Action	143
(d) Relation of Vitamin B ₁ to Other Vitamins Hormones and Minerals	144
16 Avitaminosis and Hypovitaminosis	146
(a) Clinical Test Methods	147
17 Hypervitaminosis	149
18 Requirements	150
Vitamin B₂—Riboflavin	153
1 Nomenclature and Survey	153
2 Chronology	154
3 Occurrence	154
4 Isolation	155
5 Properties	156
6 Chemical Constitution of Vitamin B ₂ Degradation Reactions	158
7 Synthesis of Vitamin B ₂ and Other Flavins	163
8 Industrial Methods of Preparation	170
9 Flavin Enzymes	171
(a) Enzyme Systems Containing Riboflavin	171
(b) Coenzymes Containing Riboflavin	177
(c) Mechanism of the Coenzyme Action	180
10 Specificity	181
11 Determination	182
(a) Physical Methods	182
(b) Biological Methods	183
(c) Biochemical Methods	184
12 Standards	184
13 Physiology of Plants and Microorganisms	185
14 Animal Physiology	186
(a) General Physiology Metabolism and Mechanism of the Vitamin B ₂ Action	186
(b) Relation of Vitamin B ₂ to Other Vitamins Hormones and Minerals	189
15 Avitaminosis and Hypovitaminosis	190
(a) Clinical Test Methods	192
16 Hypervitaminosis	193
17 Requirements	193
Vitamin B₆—Pyridoxin	197
1 Nomenclature and Survey	197

CHAPTER

PAGE

2	Chronology	194
3	Occurrence	198
4	Isolation	199
5	Properties	199
6	Constitution	200
7	Synthesis	204
8	Industrial Methods of Preparation	209
9	Biogenesis	209
10	Specificity	209
11	Determination	210
	(a) Chemical Methods	210
	(b) Biological Methods	211
12	Standard	211
13	Physiology of Plants and Microorganisms	212
14	Animal Physiology	212
15	Avitaminosis and Hypovitaminosis	213
	(a) Clinical Test Methods	215
16	Hypervitaminosis	215
17	Requirements	216
Nicotinic Acid—Nicotinamide		219
1	Nomenclature and Survey	219
2	Chronology	220
3	Occurrence of Nicotinic Acid and of Nicotinamide	221
4	Isolation of Nicotinic Acid and of Nicotinamide	221
5	Properties of Nicotinic Acid and of Nicotinamide	222
6	Constitution of Nicotinic Acid and of Nicotinamide	223
7	Synthesis	224
8	Industrial Methods of Preparing Nicotinic Acid and Nicotinamide	225
9	Biogenesis of Nicotinic Acid	226
10	Enzyme Systems Containing Nicotinamide	227
11	Coenzymes Containing Nicotinamide	229
	(a) Codehydrogenase I	229
	(b) Codehydrogenase II	234
12	Mechanism of the Nicotinamide Coenzyme Action	238
13	Specificity of Nicotinic Acid and Nicotinamide	239
14	Determination of Nicotinic Acid and Nicotinamide	240
	(a) Chemical Methods	240
	(b) Biochemical Methods	242
	(c) Biological Methods	242
15	Standard of Nicotinic Acid and Nicotinamide	243
16	Physiology of Plants and Microorganisms	243
17	Animal Physiology	243
18	Avitaminosis	246
	(a) Clinical Test Methods	247
19	Hypervitaminosis	249
20	Nicotinic Acid Requirements	250

CHAPTER	PAGE
Pantothenic Acid	253
1 Nomenclature and Survey	253
2 Chronology	254
3 Occurrence	255
4 Isolation	255
5 Properties	257
6 Chemical Constitution	257
7 Synthesis	260
8 Industrial Methods of Preparation	262
9 Biogenesis	263
10 Specificity	263
11 Determination	265
12 Standards	266
13 Physiology of Plants and Microorganisms	267
14* Animal Physiology	268
15 Avitaminosis and Hypovitaminosis	268
(a) Clinical Test Methods	270
16 Hypervitaminosis	270
17 Requirements	270
Inositol	275
1 Nomenclature	275
2 Chronology	275
3 Occurrence	275
4 Isolation	276
5 Properties	277
6 Chemistry	277
7 Industrial Methods of Preparation	277
8 Biogenesis	277
9 Specificity	278
10 Determination	278
11 Physiology of Plants and Microorganisms	278
12 Animal Physiology	279
13 Avitaminosis	280
14 Hypervitaminosis	280
15 Requirements	280
Para amino-benzoic Acid	283
1 Nomenclature and Survey	283
2 Chronology	283
3 Occurrence Isolation and Properties	284
4 Determination	284
(a) Chemical Method	284
(b) Biological Methods	284
5 Physiology	
6 Avitaminosis	

CHAPTER	PAGE
7 Hypervitaminosis	286
8 Requirements	286
Vitamin C -Ascorbic Acid	289
1 Nomenclature and Survey	289
2 Chronology	290
3 Occurrence	291
4 Isolation	292
5 Properties	294
6 Constitution	295
+ 7 Synthesis	301
8 Industrial Methods of Preparation	311
9 Biogenesis	312
10 Specificity	313
11 Determination	315
(a) Physical Methods	315
(b) Chemical Methods	316
(c) Biochemical Methods	322
(d) Biological Methods	323
12 Standards	323
13 Physiology of Plants and Microorganisms	323
14 Animal Physiology	325
(a) Metabolism	325
(b) Physiological Action	326
(c) Relation of Vitamin C to Other Vitamins Hormones Etc	332
15 Avitaminosis and Hypovitaminosis	332
(a) Clinical Test Methods	334
16 Hypervitaminosis	337
17 Requirements	337
The Group of Vitamins D	341
1 Nomenclature and Survey	341
2 Chronology	342
THE CONCEPT OF PROVITAMINS D AND OF VITAMINS D	344
PROVITAMINS D	345
3 Occurrence	345
4 Isolation	347
5 Properties	348
6 Chemical Constitution	350
7 Synthesis	360
8 Industrial Methods of Preparation	365
9 Biogenesis	366
10 Determination	366
(a) Physical Methods	367
(b) Chemical Methods	367

CHAPTER	PAGE
CONVERSION OF PROVITAMINS D TO VITAMINS D	368
11 Process of Activation	368
12 Mechanism of Activation	371
13 Chemistry of Activation Products	375
VITAMINS D	383
14 Occurrence	385
15 Isolation	387
16 Properties	389
17 Chemical Constitution	391
18 Synthesis	402
19 Industrial Methods of Preparation	402
20 Biogenesis	403
21 Specificity	406
22 Determination	412
(a) Physical Methods	412
(b) Chemical Methods	412
(c) Biological Methods	414
23 Standards	416
24 Metabolism	417
25 Physiological Action	419
26 Relation to Other Vitamins and Hormones	426
27 Hypovitaminosis and Avitaminosis	427
(a) Clinical Test Methods	428
28 Hypervitaminosis	429
29 Requirements	430
The Group of Vitamins E	435
1 Nomenclature and Survey	435
2 Chronology	436
3 Occurrence	437
4 Isolation	438
5 Properties	439
6 Chemical Constitution	440
7 Synthesis	445
8 Industrial Methods of Preparation	448
9 Biogenesis	448
10 Specificity	449
11 Determination	451
(a) Physical Methods	451
(b) Chemical Methods	452
(c) Biological Methods	454
12 Standards	455
13 Physiology of Plants and Microorganisms	455
14 Animal Physiology	456
(a) Metabolism of Vitamins E	456

CHAPTER	PAGE
3 The Essential Fatty Acids as Vitagens	532
4 Occurrence	532
5 Isolation	533
6 Properties	534
7 Chemical Constitution	534
8 Synthesis	535
9 Biogenesis	535
10 Specificity	536
11 Determination	536
(a) Chemical Methods	536
(b) Biological Methods	537
12 Standards	537
13 Physiology	537
14 Deficiency Syndrome	539
(a) Clinical Test Methods	539
15 Requirements	539
ESSENTIAL AMINO ACIDS	540
ESSENTIAL CARBOHYDRATES	542
CHOLINE AND RELATED COMPOUNDS AND THE ESSENTIAL TRANSFERABLE METHYL GROUP	543
1 Chronology	543
2 The Active Compounds and Their Properties	544
3 Pathological States Caused by a Deficiency of the Active Compounds	544
4 Specificity Studies	545
5 The Physiological Action of the Active Compounds	546
6 Requirements	548
ESSENTIAL ORGANIC SULFUR CONTAINING COMPOUNDS	549
Patent Index	553
VITAMINS GENERAL	553
Vitamins A and D	561
Distillation Procedure	566
VITAMINS A	
Provitamins A	569
Isolation	570
Synthesis	571
Analysis	572
Derivatives and Utilization	572
VITAMIN B COMPLEX	573
VITAMIN B ₁ —THIAMIN	
Isolation	575
Synthesis	576
Synthesis of the Thiazole Part	577

Patent Index (<i>contd.</i>)	PAGE
Synthesis of the Pyrimidine Part	579
Derivatives and Utilization	580
VITAMIN B ₂ —RIBOFLAVIN	
Isolation	580
Synthesis	581
Intermediates for the Synthesis	582
Derivatives and Utilization	582
VITAMIN B ₆ —PYRIDOXIN	584
VITAMIN B ₁₂	584
NICOTINIC ACID	585
PANTOTHENIC ACID	586
INOSITOL	587
VITAMIN C	
Isolation	587
Synthesis	588
Intermediates for the Synthesis	590
Derivatives and Utilization	591
VITAMINS D	
Provitamins D	594
Conversion of Provitamins D to Vitamins D	596
Vitamins D	603
Derivatives and Utilization	605
VITAMINS E	
Isolation	606
Synthesis	607
Intermediates for the Synthesis	608
Derivatives and Utilization	609
VITAMIN H	609
VITAMINS K	610
VITAMIN P	610
CHOLINE	611
Recommended Dietary Allowances	613
Author Index	615
Subject Index	659

THE VITAMINS IN GENERAL

1 Definition of Vitamins

Vitamins are organic compounds which are required for the normal growth and maintenance of life of animals, including man who, as a rule, are unable to synthesize these compounds by anabolic processes that are independent of environment other than air and which compounds are effective in small amounts do not furnish energy and are not utilized as building units for the structure of the organism, but are essential for the transformation of energy and for the regulation of the metabolism of structural units

The science of nutrition classifies the alimentary constituents (according to their function) into two different groups—energy and building unit providing food, on the one hand, and protective food on the other. The latter group comprises water inorganic substances and certain organic compounds among which are the vitamins. The definition of vitamins as given above clearly differentiates this group of nutrients from all other food constituents. Vitamins are organic compounds, and water or inorganic substances cannot be classified as vitamins. Vitamins are required for the normal growth and maintenance of life of animals including man. All known vitamins with the probable exception of vitamin D are synthesized by plants and, as far as is known, are used by them essentially for the same purposes as by man and animals. The latter, however, as a rule are unable to synthesize these compounds by anabolic processes which are independent of the environment with the exception of air. In other words the inherently available mechanisms of organo synthesis in animals and man are not provided with means to produce the vitamins. On the other hand those compounds which are produced anabolically (or catabolically) and which otherwise conform to the definition for vitamins as given above are classified as hormones. The apparent independence of cattle for certain members of the vitamin B complex and for vitamin K is due to bacterial synthesis of these vitamins in the rumen which is not an anabolic process. The production of vitamin D in the skin is not an anabolic process as defined since this process is not independent of the environ-

ment but requires energy from the outside. This energy consists of ultra violet light and is normally supplied by sunlight.

Vitamins are compounds which are effective in small amounts and occur as traces in cells and body fluids. While the maximum concentration in tissues or fluids with the exception of those which serve as storage places has not been determined for all vitamins, it is estimated that the amount of any one vitamin is less than 5 γ per gram of dry weight.¹ Vitamins do not furnish energy and are thus distinguished from the energy-bearing food constituents. The amount of vitamins needed is too small to account for even a fraction of the total energy required. Actually, minute quantities of vitamins are burned in the organism but the energy set free by this process is infinitesimal. The vitamins, furthermore, do not act as morphological or structural building units of the organism or its cells. The fact that excessive amounts of most vitamins are excreted unchanged indicates that they are not utilized as suppliers of energy or as structural building units. The vitamins are, however, essential for the transformation of energy and for the regulation of the metabolism of structural units and are functional in systems which carry out these reactions. Such systems are quite complex and are, as far as is known, enzymatic in character. These enzyme systems consist of many different components, a few of which are vitamins.

Vitamins are required by animals including man, as has been stated previously. As long as at least one animal species is known to be unable to synthesize a particular compound that compound should be considered a vitamin provided it conforms with the definition for vitamins in other particulars. Actually it cannot be expected that all animals need exactly the same nutritional elements. In accordance with this thought it has for example been observed that the cockroach apparently does not need any vitamin A,² and it is conceivable that animals will be discovered which do not need any vitamin D. Ascorbic acid, on the other hand while apparently needed by all animals, is synthesized by many of them, whereas other animals, such as the primates and the guinea pig are dependent upon an outside source of this compound. Ascorbic acid is thus a vitamin according to the definition of this term.

The classification of the nutritional elements which exert vitamin A activity requires special consideration. Man and most animals obtain in their foods two different types of substances which bring about the same

¹ D. F. Green, *Advances in Enzymology* 1: 177 (1941).

² R. I. Bowers and C. M. McCay, *Science* 92: 291 (1940).

physiological effect Both groups belong to the same class of organic compounds namely, the carotenoids They differ from each other in that the one group contains 40 carbon atoms in the molecule, while the other group contains about 20 carbon atoms The first group of substances is converted metabolically into the second group in the animal organism The second group has acquired the term vitamin A while the first group has been designated as provitamin A This terminology is not in strict agreement with the definition for vitamins according to which both groups of compounds should be called vitamins The term provitamin A has however been retained in this monograph in accordance with general usage in order to avoid further complication of the vitamin terminology, since different terms have to be applied to the two different types of compounds

The definition of vitamins as given above, has not been undisputed The most severe criticism is born of the thought that the ingested essential nutrients exert no vitamin activity as such but are active only after chemical transformation into other compounds According to these views the ingested compounds should be called provitamins unless it is established that they do not undergo transformation in the body Nicotinamide and nicotinic acid according to this interpretation should be called provitamins, while nicotinamide containing coenzymes codehydrogenase I and II should be called vitamins Similarly vitamin B₁ (thiamin) and vitamin B₂ (riboflavin) should be called provitamins This definition of the term vitamin however does not appear desirable Compounds like nicotinamide and riboflavin are constituents of a number of different enzymes and a multitude of different vitamins would have to be postulated As a result it would be difficult in many cases to decide if the coenzyme or the entire enzyme system should be called a vitamin The term vitamin would be dissociated from the science of nutrition which needs a term for these compounds The term vitamin as defined on page 3 has served a useful purpose and it appears more logical to adopt a special term for enzymes which contain vitamins such as, for example the term *vita*zyme than to change radically the present day vitamin definition which has been adopted generally

There is another group of organic compounds which are protective foods They like the vitamins are required for normal growth and maintenance of life of animals including man who as a rule, are unable to synthesize these compounds by anabolic processes They are also essential for the transformation of energy and for the regulation of the metabolism of structural units These compounds differ from the vitamins in that they also

act as suppliers of energy or as structural building units. In view of the similarity of these compounds to the vitamins but in recognition of the fact that they differ in one important functional aspect from them it is suggested that this class of compounds be called *vitagens*. This term is broad and emphasizes that the compounds of this class are concerned with the production and maintenance of life. It is recommended that this terminology be adopted until the time when more precise information is available concerning the physiological action of these compounds and the vitamins.

The number of compounds which should be classified as vitagens is unknown. There is ample evidence that they include the essential fatty acids and the essential amino acids. There may be some essential carbohydrates which conform to the vitagen concept. Choline and related compounds which supply the essential transferable methyl group are vitagens and it is suspected that some organic sulfur containing compounds will be discovered which belong to this group. The actual vitagen nature of all these compounds, however, has not been demonstrated. The essential fatty acids are structural units in many phospholipids and their physiological action appears to be primarily a regulatory one. Some of the essential amino acids have been shown to be structural building units for tissue and cellular and intracellular fluid constituents but are also constituents of enzyme systems involved in the metabolism of energy bearing foods. For example the essential amino acids lysine, histidine, tryptophane, phenyl alanine and arginine are constituents³ of the apoenzyme of a riboflavin enzyme system, and the possibility remains that some other essential amino acid is present in this system. Choline and other compounds which provide an essential transferable methyl group for the anabolism of the structure of the organism also act as regulators, for example, in the distribution of fat.

A dietary deficiency of any one of the vitagens gives rise to specific clinical symptoms which are similar to those encountered in vitamin deficiencies. Obviously, they might be considerably complex since the vitagens have the double function of providing energy or building units on the one hand, and of being concerned with the transformation of energy or the regulation of the metabolism of structural units on the other hand. A deficiency of any one of the essential amino acids which are constituents of a riboflavin enzyme system should eventually cause the occurrence of the same syndromes which are observed during times of riboflavin defi-

³ R. Kuhn and P. Desnuelle, *Ber.* 70 1907 (1937)

ciency Whether or not that is actually the case cannot be stated It might be expected that during times of a vitamin deficiency the vitamin would be liberated in the organism from the structural materials and utilized functionally This however, does not seem to be the case Thus a deficiency of choline in the diet becomes apparent from a disturbance of the distribution of fat in the liver in spite of the presence of very large amounts of choline in the phospholipids of the body ⁴

2 History of the Discovery of the Vitamins⁵

Generally speaking three distinctly different periods in the history of vitamins can be differentiated First there was the period which is characterized by the recognition of the existence of nutrient materials different from those which are needed only for the maintenance of the energy and building unit supply This period actually began many centuries ago but became a definite science at the turn of this century The second period is that of the isolation of a great number of vitamins in pure form and the elucidation of their chemical structure which culminated in the synthesis of various vitamin compounds This period started about in the middle of the 1920's when the first vitamins were obtained in crystalline form The last period is characterized by the recognition that various compounds which were previously known to exert beneficial effects in the growth of lower organisms such as yeast and bacteria are also necessary for man and animals Simultaneously an earnest effort is being made to understand the physiology and the mechanism of the vitamin action which has resulted so far in the recognition of the part which various vitamins, especially those of the B complex play in a number of different enzyme systems

Diseases of the human organism caused by a deficiency of vitamins are probably as old as the human race Among the uncovered skeletons of prehistoric man are some which show definite signs of rickets (vitamin D deficiency) and of scurvy (vitamin C deficiency) The symptoms of these diseases and of beriberi (vitamin B₁ deficiency) and of night blindness (vitamin A deficiency) were known to the physicians of ancient days and were described in various manuscripts which were written in the first thousand years A D With the exception of a remedy for night blindness no effective therapeutic methods were known at that time for curing or pre

⁴ F. X. Aylward, H. J. Channon and H. Wilkinson *Biochem J* 29 169 (1935)

For more details of the historical development see H. C. Sherman and S. L. Smith *The Vitamins* New York 1931 Medical Research Council *Vitamins A Survey of Present Knowledge* London 1937
⁵ v. Brunst *Mhch Med Wochschr* 84 223 (1937)

tional constituents. He carried out experiments on rats by supplying them with a diet consisting of purified carbohydrates, fats, proteins, inorganic material and water. The animals did not flourish, but growth resulted when 'astonishingly' small amounts of milk were added to the diet (similar to the earlier but less exact experiments of Lunn). In place of milk, the alcohol or ether soluble fraction of milk was found to bring about the same effect. In 1909 Stepp observed that similar growth promoting substances are present in bread. Pikelharing had already emphasized in 1905 in a paper which was overlooked for many years that only very small amounts of these substances present in traces in milk and probably in all sorts of foodstuffs, both of vegetable and animal origin, are necessary to keep mice alive.

Funk in 1912 reviewed the then existing knowledge of the diseases caused by nutritional errors. They were beriberi and scurvy and possibly also rickets, sprue and pellagra. Funk was apparently the first to recognize pellagra as a nutritional deficiency disease. The etiology of sprue is still unknown today. Funk attempted to isolate the compound which prevents beriberi and concluded that it is, *chemically speaking*, an amine. Funk did not succeed in isolating the pure substance but obtained nicotinic acid as a by product which he showed to have some slight beneficial growth effect. (Today we recognize nicotinic acid as the pellagra preventive compound.) In systematizing the knowledge of the nutritional elements other than carbohydrates, fats, proteins, inorganics and water, Funk called this new class of compounds 'vitamines', a term which was later changed to 'vitamins' (see under Nomenclature of the vitamins, page 12). Thus the first period of the history of vitamins in which the existence of the vitamins was recognized and proved is concluded.

In the second period of vitamin research various vitamins were isolated in the pure state, their chemical constitution established and their chemical synthesis achieved. This development was made possible by using experimental animals as tools in order to determine the presence and concentration of vitamins and for studying and differentiating the symptoms caused by special diets deficient in various constituents. The isolation, the determination of the chemical structure and the synthesis of the vitamins will be discussed later. At this point the historic development leads to a special history of each single vitamin. This will be presented in the discussion of each vitamin under "Chronology". In this period the following vitamins were elucidated: vitamin A (the compound which prevents night blindness), vitamin B₁ (the anti beriberi vitamin), vitamin B₂, vitamin B₆, vitamin C (the anti scurvy substance), vitamin D (the anti rickets com

pound), vitamin E and vitamin K. The existence of other vitamins was postulated during this period but general knowledge did not proceed further than the recognition of the existence of further vitamins and the determination of more or less specific deficiency symptoms.

The third period in the history of vitamin research proceeds along somewhat different lines of thought. While in the second period the vitamin was isolated by tedious concentration procedures using the experimental animal as the tool for the determination of the progress made, a different course was followed in the next period. This originated as a development of the study of the nutritional elements necessary for microorganisms, especially yeast and certain bacteria. J. von Liebig already had observed in the last century that yeast cannot grow properly unless the culture medium contains, besides the known and accepted nutritional elements some growth stimulating material which was found to be present in meat extracts. This unknown substance was later called *bios* and proved to be a mixture of substances. The first compound isolated from *bios* concentrates was inositol (1928) which Woolley identified in 1940 as a vitamin. Elvehjem found that the *bios* factor nicotinic acid cured blacktongue, a deficiency disease of dogs. This was soon followed by the isolation of nicotinic acid from crude concentrates of the anti blacktongue factor. Almost immediately the effectiveness of nicotinic acid in the cure of human pellagra was discovered. Several other vitamins were discovered in the same way. The yeast growth factor, pantothenic acid, proved to be the chicken antidermatitis vitamin. biotin was found to be identical with the postulated vitamin H and *p*-amino benzoic acid the growth factor for *Clostridium acetobutylicum* was shown to exert vitamin activity for man and animals. This development is not necessarily terminated as yet since further unknown growth factors for microorganisms apparently exist which may prove to be identical with vitamins for the animal organism.

As in the case of other natural sciences the first step in vitamin research is to recognize the various members. The next step is to study the physiological action and the final step is to elucidate the mechanism of the action. It seems that the main task of the recognition of vitamins has been essentially accomplished. No doubt the list of vitamins is not complete as yet and considerably more work needs to be done before all the vitamins will be recognized. Nevertheless it appears that those vitamins which cause the most obvious deficiency diseases are known. While Lunin in 1881 and Hopkins in 1906 failed to keep animals alive on a diet composed of the then known nutritional elements the situation is quite different today. It has been possible in a number of different laboratories to raise experimental

animals, rats for example on a completely synthetic diet, which includes all the known vitamins, and to keep the animals alive over a period of several generations. Simultaneously essential work is in progress for clarifying the physiological behavior of the vitamins in the organism. Once the reactions which are carried out by the vitamins in the organism are known attempts can be made to clarify the mechanism of this action. The gross physiological effect of each vitamin is evident from the symptoms caused by the deficiency of the vitamin. But these clinical symptoms may be of secondary nature and the elucidation of the primary reaction is quite difficult and requires new and special techniques (see under Physiology of the vitamins page 26). This work has shown that the vitamins are essential participants in the metabolism of energy bearing food of minerals and of water. The proof is in some cases a direct one in other cases indirect and needs confirmation. The mechanism of the vitamin action has been traced definitely for three vitamins of the B complex, namely for vitamins B₁, B₆ and nicotinic acid, which were found to take part in enzyme systems which are concerned with the processes of carboxylation and decarboxylation and by transportation of hydrogen with the oxidation-reduction mechanism.

3 Nomenclature

Hopkins⁶ (1906-1912) called the nutritional elements which are required by the animal organism in addition to the carbohydrates, fats, proteins, salts and water, accessory factors. In systematizing the knowledge of these accessory factors Funk⁷ in 1912 proposed the generic term *vitamine* because these compounds are essential to life (the Latin term *vita* meaning life) and because he believed the anti-beriberi compound to be an amine. Funk⁸ differentiated an anti-beriberi *vitamine*, an anti-scurvy *vitamine*, an anti-rickets *vitamine*, etc. according to the diseases which occurred during the respective nutritional deficiencies. No statement could be made at that time as to whether these diseases are caused by a deficiency of just one compound or of a multitude of compounds. Osborne and Mendel,⁹ and McCollum and Davis¹⁰ in 1915 distinguished two types of accessory factors by difference of their solubility and called them Fat Soluble A and Water Soluble B. Fat soluble A was shown to cure a

⁶ I. C. Hopkins *Analyst* 31: 385 (1906); *J. Physiol.* 49: 475 (1911).

⁷ C. Funk *J. State Med.* 20: 341 (1912).

⁸ C. Funk *Die Vitamine* Wiesbaden 1914.

⁹ T. B. Osborne and L. B. Mendel *J. Biol. Chem.* 20: 373 (1911).

¹⁰ E. V. McCollum and M. Davis *Ibid.* 23: 181 (1915).

nutritional eye disease (the symptoms of which are keratomalacia) and to be necessary for growth of experiments. A deficiency of water soluble B produced beriberi. The terminology did not include a term for the anti scurvy compound¹¹ in 1920 proposed to combine the previously suggested to drop the terminal 'c' of vitamine to the generic term vitamin and call the fat soluble growth and eye factor vitamin A, the water soluble anti beriberi compound vitamin B and the anti scurvy factor vitamin C. This suggestion has generally been adopted by all workers in the field and the more recently discovered vitamins have been added to the list by using the letters of the alphabet consecutively. Thus McCollum called the antirachitic compound vitamin D. The anti sterility compound was called vitamin E etc. The vitamins G, H and I were added later to the list of vitamins. The blood coagulation vitamin was called vitamin K because the term Koagulation is spelled with 'K' in the German language. The postulated lactation vitamin is called vitamin J and the compound which prevents excessive permeability of cells is vitamin P.

Considerable discussion arose from time to time as to whether or not the generic term vitamin should be maintained or changed to a term which would not indicate any relation to chemical or physiological properties of the compounds. The term accessory factors of foods was discarded because it was felt that this term is too modest since the compounds in question are essential food factors and not merely accessory food constituents. Other terms which have been suggested are Advitant and Exogenous Hormones. Neither of these terms has however been generally accepted.

The further development of the vitamin terminology has unfortunately resulted in an illogical nomenclature which can only be understood from its historical development. What was originally called vitamin B proved to be a mixture of compounds which are referred to today as the vitamin B complex in accordance with the original nomenclature. This complex consists of an unknown number of different vitamins which have been designated arbitrarily as vitamin B₁, B₂ etc. The anti beriberi vitamin which was first discovered is called vitamin B₁. So far eight different vitamins have been given vitamin B subnumbers. One of the difficulties which arose during the course of the vitamin research was that a deficient diet gave different symptoms in various animals although it was found later that some of these symptoms were caused by the absence of the same vitamin. Thus vitamin B₂ has also been called vitamin C. Originally vitamins B

and B₄ were believed to be necessary only for the rat while vitamins B₂ and B₃ were believed to produce growth only in the pigeon

By the time that the vitamins were isolated as chemical compounds, they were given names which identified them according to the class of chemical compounds to which they belong. Thus, vitamin B₁ is called 'thiamin,' vitamin B₂ 'riboflavin,' vitamin B₆ 'pyridoxin,' etc. While the research on the isolation of the vitamins B₃, B₄ and B₅ was progressing only slowly, other vitamins were discovered through experimental work with different organisms. Thus nicotinic acid, pantothenic acid and biotin which were known to be growth stimulants for yeast were also found to be vitamins for the animal organism. It has subsequently been discovered that pantothenic acid is probably identical with vitamin B₃ and nicotinic acid with vitamin B₅. Biotin, glycine and arginine, on the other hand, appear to be present in what was originally called vitamin B₄.

While the original vitamin B has been shown to consist of a number of chemically and physiologically different compounds which were differentiated by subnumbers, the fat soluble vitamins A, D, E and K each have been found to occur naturally not as single compounds but as mixtures of compounds each of which exerts the same physiological action but differs from the others slightly in its chemical constitution. Thus there exist two or three different vitamins A, at least six vitamins D, three vitamins E and two vitamins K. They are called vitamins A, D, E and K and are differentiated by subnumbers. Thus, for example, vitamin A₁ exerts essentially the same physiological effect as vitamin A₂. On the other hand, in the water soluble class of vitamins each subnumber indicates a physiologically different vitamin with an entirely different mechanism of action as has been stated before.

The vitamins have been classified as biocatalysts together with the hormones and enzymes. Instead of the term 'biocatalysts' the term 'ergins' has been proposed by Ammon and Dirscherl¹². On the other hand Euler¹³ advocated the term 'ergons' for the vitamins, hormones and those coenzymes which contain as part of their structure vitamin or hormone molecules. Such coenzymes have also been called 'vitazymes' and 'hormozymes'.

4 List of the Vitamins

Two classes of vitamins are differentiated today, namely, those which have been identified and those which have not been definitely identified.

¹² R. Ammon and W. Dirscherl, *Ferment Hormone Vitamine*, Leipzig 1938.

¹³ H. v. Euler, *Arkiv Kemi Mineral Geol.* A11 No 12 (1934) B11 No 45 (1934) B12 No 11 (1935).

By identification is meant the recognition of a vitamin as a chemical compound the physical properties of which have been established

THE IDENTIFIED VITAMINS

Vitamin	Name
The Group of Vitamins A	
Vitamin A	Axerophthol
Vitamin A ₂	
Vitamin A ₃ ?	
Vitamin B ₁	Thiamin
Vitamin B ₂	Riboflavin
Vitamin B ₆	Pyridoxin
Nicotinic Acid (Vitamin B ₃ ?)	Vitamin B Complex
Pantothenic Acid (Vitamin B ₅ ?)	
Inositol	
p Amino Benzoic Acid	
Vitamin C	Ascorbic acid
The Group of Vitamins D	
Vitamin D ₂	Vitamin D ₂ calciferol
Vitamin D ₃	
Vitamin D ₄	
Vitamin D ₅	
Vitamin D ₆	
The Group of Vitamins E	Tocopherols
α Tocopherol	
β Tocopherol	
γ Tocopherol	
Vitamin H	Biotin
The Group of Vitamins K	
Vitamin K ₁	
Vitamin K ₂	
Vitamin P	Citrim

THE NON IDENTIFIED VITAMINS

Vitamin B ₇ (Pantothenic Acid ?)	Group of Vitamins L (Lactation Vitamin)
Vitamin B ₈ (Biotin Arginine Glycine ?)	Vitamin L ₁
Vitamin B ₉ (Nicotinic Acid ?)	Vitamin L ₂
Vitamin B ₁₀ —Vitamin I	Vitamin M
Vitamin B ₁₁ (Adenylic Acid)	Factor T
Vitamin B	Factor U
Vitamin B _p (Anti Peroxis Vitamin)	Folic Acid
Vitamin J	Grass Juice Factor

A list of the vitamins is as follows

THE VITAMINS

Essential fatty acids
Essential amino acids
Essential carbohydrates

Choline and related compounds and the
essential transferable methyl group
Essential organic sulfur containing com-
pounds

5 Occurrence

The natural distribution of the vitamins is discussed in this monograph separately in the vitamin chapters under the heading of occurrence. Generally speaking vitamins occur in plant materials and are found in the animal organism only as a result of food intake or of the anabolic activity of micro organisms living in the intestinal tract. The vitamin content of various sources is of eminent importance for the proper selection of food for man and animals and is often the determining factor in the commercial preparation of vitamin concentrates. Nevertheless, quantitative data as to the vitamin content are given only occasionally in this monograph. For practical purposes it is not possible to assign definite data to the vitamin content of foods because the vitamin content is only to a limited extent a function of the vitamin concentration in the fresh vegetable in the fresh fruit etc. The amount of most vitamins contained in food after cooking or preservation procedures differs considerably from that of the untreated food. In most cases the vitamin content decreases during cooking heating and washing procedures while in some instances the amount of available vitamins increases by liberation from protein material.

Among the many other factors which influence the vitamin content of vegetable foods are the species the time of harvesting and the soil. And the potency of animal foods depends somewhat upon the vitamin supply in the animal's diet.

Extensive studies have been made to learn the vitamin content of practically all foods and foodstuffs treated and untreated. The available information has been collected in a number of monographs¹⁴ which are recommended as an excellent guide for the approximate vitamin potency of foodstuffs.

¹⁴ F. P. Daniel and H. F. Munsell *United States Department of Agriculture Miscellaneous Publication No. 27* (1937)
I. B. Booher, E. R. Hotzler and E. M. Heaton *United States Department of Agriculture Circular No. 634*
(1942). M. A. B. Finsen and M. H. Rose *Nutrition Abstracts and Reviews* 9: 73 (1939). A. L. Bacharach *Ibid.* 10: 43 (1941). H. A. Wainman and C. A. H. Lyon *The Vitamin Content of Meat*, Burgess Publ. Co. Minneapolis, 1941.

6 Isolation

The first step in the recognition of a new vitamin is to produce a nutritional deficiency in an experimental animal. The next step consists in the isolation of the compound which exerts vitamin activity. The procedure of isolating the unknown vitamin is very tedious and requires in many cases a million fold concentration of the original material. In the beginning of the history of vitamin research no effective methods were available for the isolation of compounds which occur in such small amounts and in mixtures with such enormous quantities of other substances. New methods therefore, had to be devised and many old methods which were effective enough for the isolation of other compounds had to be modified and refined. The most important new methods are the chromatographic adsorption technic and the high vacuum molecular distillation. Both of these methods have been developed to such an extent that they are now used for the isolation of many vitamins in the pure form and have found commercial applications.

Under the heading Isolation the methods used for the isolation of each vitamin are discussed separately in the respective chapters.

7 Chemical Constitution

Once a vitamin has been isolated in the pure or essentially pure state efforts are made to elucidate the chemical constitution. The methods for the analysis and determination of the structure are principally the same as those which have been used for the determination of the structure of many organic compounds of non vitamin character. They consist essentially in the degradation of the unknown compound to smaller molecules until derivatives of known structure are obtained. The structure of the original vitamin molecule is then reconstructed by skilful organic chemical reasoning.

Since each of the vitamins has a structure which is entirely different from that of any other vitamin it is necessary to present the work carried out in determining the chemical constitution of the vitamins separately for each vitamin. Therefore the results of the experimental work which led to the postulation of the structure of the compound are reported under the individual vitamins.

8 Synthesis

The normal consequence of a postulation of the constitution of a vitamin is a challenge to synthesize the vitamin. Actually all vitamins which

have been isolated have been synthesized with the exception of biotin (for which no structural formula has been suggested as yet due to the fact that sufficient amounts of the pure vitamin have not been available for chemical studies) A total synthesis is regarded as the final proof for the formula which has been postulated on the basis of degradation reactions The methods used for the synthesis of the various vitamins are presented in special chapters No total synthesis of any one of the vitamins D has been accomplished as yet, but some of the provitamins D have been obtained synthetically by hemi synthesis from various sterols

9 Industrial Methods of Preparation

An important feature in the development of vitamin research has been the availability of the vitamins to the clinician and practitioner for studies on experimental animals, for use in human therapy and finally for incorporation into foodstuffs for man and animals The first industrial methods which were used in the production of vitamin preparations for the public were extractions from natural sources Originally crude concentrates were offered to the trade but recently the methods of isolation have been refined to such a degree that it is possible to offer commercially practically all the vitamins in essentially pure state Besides the isolation of the natural compound synthetic methods of preparing the vitamins have been studied and developed to such an extent that it is now more economical to produce most vitamins on a commercial scale by synthesis than by extraction from natural sources

The methods used in industry for the manufacture of vitamin preparations are known in general but the details are usually kept secret Under the heading of industrial methods of preparing vitamins the procedures used commercially are briefly reviewed for each vitamin The result of industrial research and development work is reflected in patents which have been taken out to protect the manufacturers and the public, both of whom are interested in obtaining the best possible products and preventing unskilled and dishonest manufacturers from exploiting the market ¹⁵ A list of the issued patents from the main industrialized countries United States of America, Great Britain, Germany and France will be found at the end of this monograph arranged according to subject In isolated cases, patents issued in Switzerland Holland Belgium, Canada Japan and Russia have also been incorporated

¹⁵ A C Connolly *Science* 86 383 (1937)

10 Biogenesis

The methods used in the plant organism for synthesizing vitamins which are complex compounds, are very interesting from a physiological standpoint. While up to the present time these methods are not known for all the vitamins and only a few theoretical approaches have been made, it is worth noting that the building principles for the synthesis of vitamins are the same as those used by the plant organism for the synthesis of many structural units. Thus we find there is a vitamin represented among practically all types of compounds synthesized in plants. Further research will elucidate the reaction mechanism used by the plant organism for the synthesis of the structural building units and hence for vitamins in particular. Ultimately, the physiologist wishes to determine why vitamins are synthesized, why they are produced only in such minute quantities and how the mechanism for synthesizing these particular compounds differs from that for other chemically very closely related substances which have no vitamin action.

11 Specificity

One of the most fascinating studies in biochemical research is the determination of the specificity of the vitamins. Generally speaking, two types of specificity must be differentiated—namely, compound specificity and species specificity. By compound specificity is meant the qualitative and quantitative differences in physiological behavior of various compounds on the same animal organism. Species specificity is defined as the difference in physiological response of one or more compounds on different species of animals. The various forms of vitamin D may serve as an illustration for both the compound and species specificity. Vitamin D₂ (activated ergosterol) and vitamin D₃ (activated 7 dehydro cholesterol) have qualitatively and quantitatively the same antirachitic activity when tested on rats. Vitamin D₄ (activated 22 dihydro ergosterol) has qualitatively the same antirachitic efficacy but quantitatively the effect is only half as great as that of the vitamins D₂ and D₃ on the molecular weight basis. This effect demonstrates compound specificity. When the same three vitamins D are tested on chicks under standardized conditions on the basis of Rat Units and compared with cod liver oil it is observed that vitamin D₃ is as active as the vitamin D in cod liver oil and this activity is arbitrarily designated as 100% activity. On the other hand when vitamin D₂ is tested under the same conditions an activity of only a few per

cent is obtained Vitamin D₂ exerts an activity of about 20% under the same conditions This effect demonstrates species specificity

One of the objects of studying the specificity of vitamins is to determine whether or not the vitamin activity is due to the structure of the entire molecule or to a special group This type of research has proved to be of extreme importance in the study of many pharmaceutical compounds A well known example in the field of chemotherapeutical compounds is the research which followed the discovery that "prontosil" counteracts streptococci¹⁶ Investigations revealed that the bactericidal action is due solely to the sulfanilamide portion of the molecule¹⁷ Research of this type in the vitamin field brought to light that the activity exerted by choline is due to the available methyl groups of the compound and resulted in the recognition of the available methyl group as a vitamin (or vitagen) factor Specificity studies on the group of vitamins K showed that while the naturally occurring vitamins K₁ and K are 2 methyl 1,4 naphthoquinone derivatives with long side chains, the vitamin activity is due only to the 2 methyl 1,4 naphthoquinone portion of the molecule

Specificity studies in other fields have revealed that it is possible to alter the properties of the active compound so as to make them more suitable for therapeutic use One of the best known examples in the hormone field is the use of methyl testosterone¹⁸ which does not occur naturally but which is effective when given by mouth¹⁹ whereas the naturally occurring testosterone is active only by injection Alterations of chemical or physical properties without alteration of physiological action in the vitamin field are of importance, for example in order to make fat soluble vitamins water soluble or water soluble vitamins fat soluble The most important practical problem of this type concerns vitamin K K avitaminosis occurs in patients with obstructive jaundice due to the fact that the bile does not reach the intestinal tract and thus prevents the vitamin from being absorbed On the other hand water solubilized forms of vitamin K do not require the presence of bile for absorption from the intestinal tract and are thus active as such when taken orally

From the vast amount of work which has been done to elucidate the compound specificity of the vitamins it can be concluded that the physiological action of vitamins is very specifically a function of the entire molecule In the class of water soluble vitamins it is observed that lower or higher

¹⁶ C. Domagk *Deut. Med. Wochenschr.* 61 250 (1935)

¹⁷ J. Tréfouël, J. Tréfouël, F. Nitti and D. Bovet *Compt. rend. soc. biol.* 120 756 (1935)

¹⁸ I. Ruzicka, M. W. Goldberg and H. R. Rosenberg *Helv. Chim. Acta* 18 1487 (1935)

¹⁹ K. Miescher and I. Tschopp *Schweiz. Med. Wochenschr.* 68 1258 (1938) *I. G. L. o. S. B. i. Med. J.*

homologs have either much lower activity than the vitamin proper or have no vitamin activity whatsoever. This is not the case in the class of fat soluble vitamins. Actually they occur naturally as a mixture of various homologs. Geometrical isomers of the vitamins are usually considerably less active than the vitamin itself. Thus activated *epi* 7 dehydro cholesterol is only about one tenth as active as activated 7 dehydro cholesterol. Another example of the high specificity of the vitamins is the specific effect of *d* ribose in the vitamin B₂ molecule. This sugar compound cannot be replaced by any other sugar without essential loss of activity.

Under the heading Specificity the results obtained from the experimental studies and the conclusions reached will be presented separately for each vitamin.

12 Determination

One of the most fruitful developments in the biological field has been the study of methods for measuring the potency of vitamin preparations. There are principally a number of different procedures for the determination of vitamins. These comprise physical, chemical, biochemical and biological methods. Generally speaking any method has to be investigated thoroughly in order to determine the specificity afforded by the test, the types of compounds which inhibit an evaluation of the procedure and finally the sensitivity of the procedure.

Among the physical methods for the determination of vitamins spectroscopic studies are outstanding. These include determinations of the absorption spectrum in the ultraviolet or visible light region. The fluorescence spectrum is used successfully in specific cases. These tests are based on the presence of a specific chemical group in the vitamin molecule. Therefore this procedure cannot be specific for any vitamin since the same physical phenomenon is given by any other chemical compound with the same characteristic group. Nevertheless the physical methods are quite accurate when appropriately used and are in general more rapid than other methods.

The basis for all determinations of absorption spectra is Beer's law

$$I = I_0 10^{-\epsilon c d}$$

In this formula I_0 stands for the intensity of the incident beam, I for the intensity of the transmitted beam, c for the concentration (expressed in mols per liter), d for the depth of the solution (in cm) and ϵ for the extinction coefficient. There is however no uniformity in the use of the units.

As long as a compound has not been isolated in the pure form merely transmissions of solutions of known concentration are plotted and expressed as $\log I_0/I = \epsilon c d$. The adoption of a solution of 1 cm depth standardized to contain 1% of the substance and expressed as *extinction* $E_{1\text{cm}}^{1\%} = \log I_0/I$ is a frequently used refinement in the study of absorption characteristics. The *absorption constant* K has also frequently been used and is defined in the formula

$$I = I_0 e^{-K c d}$$

(e is the basis of the natural logarithm) When the molecular weight of a compound is known, the extinction of the absorption per mol is measured and expressed according to Beer's law either as

$$\epsilon = \frac{1}{c d} \log \frac{I_0}{I}$$

or

$$A = \frac{2.3}{c d} \log \frac{I_0}{I} \left[\frac{10^3 \text{ cm}^2}{\text{g mol}} \right]$$

The chemical methods for the determination of vitamins are based upon certain chemical reactions which the vitamin molecule undergoes with specific reagents. Usually those types of reactions are used for the determination of vitamins which develop a color which can be measured quantitatively. Such methods have been worked out for practically every vitamin and can be carried out relatively quickly. However they have the disadvantage that the reactions are not specific for the vitamin since the same or similar color reactions are also given by a series of chemically similar and different compounds.

Biochemical methods for the determination of vitamins are defined as procedures in which the vitamin is determined by the part it takes in certain biochemical reactions. An illustration is the action of vitamin B_1 in a specific enzyme system which decarboxylates pyruvic acid. These procedures are very specific for the particular vitamin which is determined but require special skill in handling enzyme systems and are usually quite tedious. They are used in special cases with considerable advantage over any of the other procedures.

All physical chemical and to a certain extent also the biochemical methods for the determination of vitamins are valid only if their results can be related to actual biological values. Thus, the biological methods which

have generally been used as the primary tool in the discovery of the vitamins, must still be resorted to today as the final criteria for qualitative and quantitative vitamin assays and for standardization and determination of the accuracy of other methods. Biological methods are very time consuming and costly. The primary requirement is to feed a group of comparable animals which are used for the determination of vitamins a special diet which is deficient only in the vitamin to be tested and is otherwise well balanced in regard to the other vitamins and the energy and building unit bearing and protective food constituents. Multiple deficiencies, that is, the concurrence of avitaminoses caused by lack of more than one vitamin should generally be avoided in biological vitamin assays. The actual determination can be carried out either on a curative or on a prophylactic basis. The curative methods are usually preferred since it is possible to use for the actual test only those animals which have been inflicted successfully with the deficiency disease. It is often necessary to carry out simultaneously with the vitamin determination check experiments with animals which have not obtained the vitamin supplement. The highest possible degree of accuracy is achieved by comparing in parallel experiments the activity of the unknown material with the activity of a standard preparation. The results of biological assays are evaluated statistically. Mathematicians and biologists have given much thought as to the method of carrying out this statistical evaluation most efficiently in order to achieve a high degree of accuracy with a minimum of experimental animals.

A special type of biological method for the determination of vitamins is the so called microbiological method in which microorganisms are used as test objects. Thus practically all members of the vitamin B complex can be determined by the growth effect which they exert on microorganisms, such as yeast and different strains of bacteria. The amounts of vitamin required for such microbiological assays are considerably smaller than are needed in experiments with higher animals. The microbiological tests are relatively inexpensive and can be carried out in a relatively short period of time. Especially sensitive are such microbiological methods which are based not on the growth of the organism but on the action of the organism in special culture media. Thus those bacteria are especially useful which produce acids for example lactic acid. The actual determination of the vitamin effect involves in such cases a titration of the amount of acid formed. This type of biological vitamin assay has proved to be of high accuracy in the determination of pantothenic acid nicotinic acid and biotin.

The special methods which have been used and recommended for the determination of each vitamin are presented and discussed separately under each vitamin

13 Standards

Standards of vitamins are necessary for the determination of these protective food constituents and for proper dosage. Vitamins originally were defined in terms of biological units. However, as soon as a vitamin is obtained in crystallized form and is easily accessible in that form, the standard is expressed on the weight basis of the crystallized material.

The term 'biological unit' is a generic term for specific animal units such as Rat Unit, Chick Unit, Mouse Unit, etc. The biological units of non identified vitamins are of relatively uncertain definition. They represent the reciprocal of a dose which had a certain effect on a certain animal at a certain time. Thus a biological unit refers to the action of an animal while a standard based on the weight unit refers to the amount of the vitamin.

During the last two decades, national and international standards have been set up for many vitamins, especially for the non identified vitamins. By the time a vitamin is available as a stable crystallized compound a new standard is set up which defines the unit in terms of actual weight of the vitamin. Usually that amount of crystallized vitamin is defined as one unit which corresponds on a comparative basis to the effect of the original biological unit.

The standards as adopted or as recommended for adoption are presented for each vitamin separately in the corresponding chapters.

PHYSIOLOGY

The physiology of vitamins comprises a study of the vitamin action in plants and animals. Since by definition specific organic compounds which are indispensable to the animal organism are called vitamins and since the definition contains no statement concerning plants, it is obvious that the physiological action of the vitamin compounds in the animal organism is considered to be of primary importance. Nevertheless the physiological action of these compounds in plants where most vitamins are synthesized, must be incorporated in a complete study of the vitamins. Animal physiology is subdivided with respect to the action of the vitamins in the organism into (a) the metabolism of the vitamins (b) the physiological action of the vitamins (c) the mechanism of the vitamin action and (d) the relation of the vitamins to each other to hormones and minerals.

The sections on the physiology of the vitamins are presented in a somewhat different manner from that of the sections on the chemistry of the vitamins due to the different character of the subject. The knowledge of both the chemistry and the physiology is based on experimental work. There is no, or practically no, misunderstanding possible in the conclusions drawn from intelligently planned chemical experiments. This is due to the fact that chemical experiments deal generally with systems the constituents and the reactions of which are known and contain as the only unknown the vitamin. The situation is entirely different in experiments designed to elucidate the physiology of the vitamins or the mechanism of the vitamin action. Here the vitamin acts in a very complicated and largely unknown system namely, the living organism. As a result all experimental data obtained from studies on the living organism must be qualified as to the exact conditions used in the experiments. The multiplicity of possible interpretations of these results then causes difficulties. The same physiological experiment carried out under only slightly different conditions may give entirely different results. The interpretations of these results are then bound to differ markedly. By the time the knowledge of the chemistry and the physiology of the vitamins and the living organism expands it is evident that conclusions drawn from earlier experiments must often be modified due to the fact that all statements regarding physiological reactions are based on certain assumptions and hypotheses regarding the equivalency of principal physiological reactions under various reaction conditions.

The result of all these factors is that a tremendous amount of work is necessary to establish the true physiological action of the vitamins. In a few cases this has already been accomplished. In most cases a final understanding has not been reached. A review of these latter cases is extremely difficult since from the mass of data and interpretations available only a limited amount can be presented in this monograph. The discussion of the experimental data is usually followed by interpretations which are mostly subjective in nature. Efforts have been made to present the material in the form of a well rounded picture but it appears possible that just this attempt has led to the presentation of conclusions which ultimately may prove to be erroneous. A few examples of somewhat questionable views may be cited. The existence of a combined form of ascorbic acid ascorbigen is accepted as proved in at least a few instances although many references could be cited to disprove the theory of the existence of ascorbigen. The view is taken that vitamin D is less efficacious for human beings than is vitamin D₂ although statements disproving this thesis can be found

in the literature In most cases in which a distinct difference of opinion exists between various groups of workers both sides are cited

14 Physiology of Plants and Microorganisms

Most vitamins are synthesized in plants There is apparently a special mechanism for the synthesis of each of the vitamin compounds Very little is known about the physiological behavior of these compounds in plants, but it has been shown in a few isolated cases that the vitamins are essential growth promoting substances for the plant organism It is assumed that the vitamins act in the plant organism principally in the same manner in which they act in the animal organism

The information available concerning the physiological action of the vitamins in microorganisms is very meager The most interesting discovery in this field is the observation that while some microorganisms are able to provide their own needs like the plants there are others which rely upon an outside supply of the vitamins Curiously enough there are also quite a number of microorganisms which do not have the power of synthesizing the vitamins completely, but which are able to synthesize part of the vitamin molecule and are able to build up the entire molecule when the missing component is supplied

15 Animal Physiology

(a) Metabolism of the Vitamins

Vitamins are by definition compounds which cannot be synthesized in the organism of man or animals With one single exception (vitamin D) the vitamins are always supplied to the human and animal organism by oral administration as normal food constituents. In the properly functioning organism they are readily absorbed in the intestinal tract The water soluble vitamins are absorbed as such at least when they are supplied in the free form In combined form they have to be hydrolyzed to the free vitamin before absorption can take place The fat soluble vitamins on the other hand require the presence of bile for proper absorption. Following the absorption the vitamins are carried through the organism by means of the blood stream and are carried to the tissues and organs which need them.

The animal organism does not have special storage organs for the deposition of vitamins A certain level of vitamins is usually maintained in tissues and body fluids and some special organs contain increased amounts The reason for the presence of these increased amounts is not obvious,

but is believed to be a necessity for the proper functioning of that particular organ. This conclusion has been reached in recognition of the fact that during times of low vitamin intake these organs are not depleted of their vitamin content, although the actual amount is usually somewhat reduced. Simultaneously, symptoms of vitamin deficiencies occur long before the amount present in these special organs is exhausted. Thus, the amount of vitamins present in such special organs cannot be considered as a storage of the vitamin for distribution over the entire organism.

The physiological reason for maintaining a certain normal vitamin concentration in the organism is apparently a certain safety factor for times of low vitamin intake. To a limited extent the body appears to contain a self regulatory mechanism for the utilization of the vitamins. This is apparent from the fact that while the healthy organism on a normal diet excretes certain amounts of the vitamins they are not excreted or are excreted only to a limited extent at times of low vitamin intake even when abundant quantities are present in the organism.

Administration of excessive amounts of the vitamins usually causes their excretion within a relatively short time. The water soluble vitamins are excreted mainly through the urine. The fat soluble vitamins are excreted through the feces. A certain amount is destroyed in the organism. It appears that once a vitamin molecule is destroyed it is completely burned since it has not been possible so far to find any degradation products in the excretions.

In accordance with the life maintaining function of the vitamins it is obvious that these protective compounds are secreted in the nutritional materials necessary for proper growth and development of the offspring. Thus small but definite amounts of all vitamins are found in milk and eggs.

(b) *Physiological Action of the Vitamins*

The principal physiological action of vitamins has become a part of the vitamin definition. They do not furnish energy and are not utilized as building units for the structure of the organism but are essential for the transformation of energy and for the regulation of the metabolism of structural units.

Vitamins are necessary for the animal organism as a whole not for the individual cells although the reactions of the vitamins are carried out predominantly in cells. Cells deprived of vitamins can survive and multiply while the entire organism cannot.²⁰

²⁰ S. B. Wolbach, *Science* 86: 569 (1937).

The primary physiological action of most vitamins is scarcely known. The observations made in experimental studies are in many cases secondary symptoms or secondary reactions, and it is very difficult to decide in many cases whether the observed actions of the vitamins are of primary or of secondary nature. Whatever they may be it seems that, at least on the basis of the present day information, a certain difference exists between the physiological action of the water soluble and fat soluble vitamins. The fat soluble vitamins and also water soluble vitamin C apparently maintain the 'regulation of the metabolism of structural units'. From the experimental data available it may be concluded that vitamin A is concerned with the building of the cell nucleus and vitamin E with the maturation and differentiation of cells. Vitamin C enables the cells to produce supporting tissues and to maintain intercellular substances. Vitamin D plays an essential role in the process of bone calcification, which process is a deposition of calcium phosphate in bone matrix. Vitamin K maintains the level of certain structural (or at least potentially structural) building units of blood which initiate the process of blood coagulation under proper conditions. In contradistinction from the action of these vitamins, the water soluble vitamins of the "B complex" are concerned mainly 'with the mechanism of the transformation of energy'. Thus the vitamins B₁, B₂ and nicotinic acid have been shown to take part in certain reactions which regulate the carbohydrate metabolism. Vitamin B₆ is apparently involved in the amino acid metabolism.

The observations and conclusions reached from experimental data concerning the physiological action of the vitamins in the animal organism are presented separately for each vitamin in the corresponding chapters.

(c) *Mechanism of the Vitamin Action*

Ultimately the mechanism of the action of each vitamin will be understood. So far the reaction mechanism of only a few vitamins is known. The primary requisite for studies of the mechanism of the vitamin action is a proper understanding of the vitamin action as such. As has been pointed out in the previous paragraph, the physiological action of the vitamins is as a whole not properly understood. As a result the mechanism is unknown. In the few cases in which the primary physiological action of a vitamin has been elucidated efforts have been made to determine the mechanism of the vitamin action. Thus, in the cases of the vitamins B₁, B₂ and nicotinic acid of the vitamin B complex the relation of the vitamins to the carbohydrate metabolism has been the subject of many experimen-

tal studies and theoretical considerations. These have culminated in the recognition of the fact that these three vitamins are part of enzyme systems which can be essentially separated from the animal organism and can thus be subjected to experimental studies under defined conditions in contradistinction to the reactions in the organism where the conditions are not defined at least not from an experimental point of view. As a result of this the mechanism of the action of these three vitamins is at least partly understood.

The mechanism of the action of the other vitamins is not known but various theories have been presented which attempt to explain the experimentally observed phenomena.

(d) Relation of the Vitamins to Each Other to Hormones and Minerals

An integral part of the physiological action of the vitamins is their influence on the secretion of hormones and on the metabolism of inorganic compounds. However no physiological relation exists between any one vitamin and another.

Shortly after the recognition of the existence of vitamins a theory of synergism and antagonism of the vitamins was postulated. According to this theory the elimination of one vitamin from the food causes a change of the action of the remaining vitamins. Certain experimental facts have been cited to prove this conception but a logical consideration of the vitamin concept disproves the existence of this theory. If it were true that the elimination of one vitamin changes the action of the remaining vitamins it would be impossible to observe specific vitamin deficiencies. It must be remembered that the vitamin intake of the average human being changes considerably from day to day. It is quite certain that the amount of vitamins administered every day changes markedly. Thus, on one day a considerable amount of one vitamin may be consumed while the diet of the next day may not contain any of this vitamin. If the theory of synergism and antagonism of vitamins were correct each vitamin would thus exert a different type of reaction practically every day. The knowledge of the vitamin action and of the mechanism of the vitamin activity of certain members of the vitamin B complex makes it appear improbable if not impossible that one vitamin could replace another in these reactions.

Nevertheless the theory of antagonism and synergism of vitamins has been generally accepted although the experiments on which such a theory is based are not conclusive and have not been carried out under convincingly well chosen conditions. Recent carefully controlled investiga-

tions²¹ have indicated that the vitamins A, B₁, B complex and D do not act according to the postulated theory at least as long as administered in amounts which correspond to the optimal requirement of these vitamins. It has thus been shown experimentally that a definite relation of one vitamin to another does not exist.

It has been found that certain vitamins, especially vitamin C, but also vitamin A and vitamin B are able to exert in addition to their vitamin action also other therapeutic effects. Thus, vitamin C has been found to counteract intoxications caused by many organic and inorganic compounds, toxins, etc. This action is not specific for vitamin C but is a function of a particular group in the molecule. Other compounds with similar chemical groups are able to exert the same or similar effects. This detoxifying action of vitamin C has also been observed in certain experimental animals when excess toxic doses of fish liver oils have been administered. The beneficial effect of vitamin C has in this case been interpreted as indicating an antagonism of vitamin C to the vitamins present in fish liver oils. Actually, however, vitamin C acts as a detoxifying agent for the harmful components of the liver oil regardless of whether or not they are vitamins.

Although the author of this monograph is of the definite opinion that the theory of synergism and antagonism of vitamins is incorrect and that such actions do not exist, theories concerning these phenomena and experimental data obtained in studies of these effects are presented in the corresponding section of each vitamin. This presentation may assist in the ultimate recognition of the non existence of functional relations of one vitamin to another.

In contradistinction to the relations of vitamins to each other, ^{(2) has been found that} there is the possibility that vitamins may influence the secretion or action of hormones. Since the vitamins maintain animal life, they may as part of their action influence secretions within the organism. Thus, a relation of vitamins to hormones is not illogical although not necessary a priori. Thus, it can be generally observed that in animals which have been deprived of vitamins for a long period of time, not only the entire organism suffers but that specifically the glands lose vitality and as a result of this the effective secretion is reduced. This has been especially obvious, for example in the case of the germinal glands. In addition to this general effect of vitamins on the secretion of hormones, there has also been postulated a theory of synergism and antagonism of vitamins to hormones. In a few isolated cases indications for such relations have been observed. Thus

²¹ P. E. Samola, *Ber. ges. Physiol. exptl. Pharmacol.* 83: 312 (1936). A. Scheunert, *Naturwissenschaften* 28: 297 (1940).

there is what has been interpreted as a synergism between vitamin C and the hormones of the adrenal medulla and cortex. An antagonism between vitamin C and the thyroid hormone has also been postulated. It is extremely difficult to prove or disprove experimentally the existence of relations of this type between vitamins and hormones. For a long time such a relation had been assumed to exist between the hormone of the thyroid gland and vitamin D but more thorough studies have indicated that such a relation actually does not exist.

It has already been stated under Physiological Action of the Vitamins (page 27) that they are concerned with the regulation of the metabolism of the structural units. Thus a relation of the vitamins to those inorganics which act as structural building units is obvious. In addition a relation of the vitamins to the functional inorganic building units of the organism exists. Such a relation is however indirect in the sense that certain inorganics act vitamin like in addition to the vitamins and in cooperation with them. An antagonism or synergism is not known. As an example of such a cooperative action may be cited the essential action of manganese in enzyme systems which contain vitamin B₁ and the action of copper in ascorbic oxidase.

PATHOLOGY

The study of the pathological effects caused by vitamins comprises a study of the effects caused by an administration of insufficient amounts of the vitamins and of excess doses. The symptoms caused by the former conditions are called avitaminoses and hypovitaminoses whereas the symptoms caused by the latter conditions are called hypervitaminoses.

16 Avitaminosis and Hypovitaminosis

During times of vitamin deficiencies two types of clinical symptoms can be observed namely specific deficiency syndromes and general non specific effects. The latter comprise what is generally known as ill health and constitute minor aches and a feeling of discomfort. In experimental animals a premature aging also is observed. Such conditions are usually not specific for a particular vitamin but may be caused by a deficiency of practically any vitamin. The specific deficiency syndromes are classified according to the severity of the cases into hypovitaminosis and avitaminosis. The state of hypovitaminosis is characterized by a partial vitamin deficiency, that is a state at which a vitamin is administered in suboptimal amounts. Under these circumstances the general health of the organism

decreases and specific deficiency syndromes set in. These are usually not very severe and can be cured easily by the administration of the proper vitamin. A total deficiency of a vitamin causes the appearance of specific clinical symptoms in a much more severe state and is called avitaminosis. These diseases can usually be cured at an early stage. Organisms which have suffered from avitaminoses for a prolonged period of time cannot always be repaired completely. The clinical symptom which remains after treatment with vitamins is called paravitaminosis.

The special symptoms of hypovitaminosis and avitaminosis in human beings and in experimental animals are described separately for each vitamin in the corresponding chapters.

(a) *Clinical Test Methods*

While it is usually easy to diagnose an avitaminosis, the recognition of a hypovitaminosis is often difficult due to the fact that the symptoms are not very specific. Methods of determining the state of hypovitaminosis are, therefore, of considerable importance. Hand in hand with this problem goes the problem of determining the average or individual requirement for a specific vitamin in order to insure the success of a prescribed vitamin therapy. Tests have therefore been developed and are being perfected in which the concentrations of vitamins in the organism can be determined quantitatively. In general, such tests deal with the determination of the vitamin in blood where a certain level usually is maintained and in the urine or feces provided the vitamin is regularly excreted. Special saturation tests are being developed for many vitamins in which either the amount of vitamin necessary or the time consumed is measured until a certain minimum concentration of the vitamin occurs in blood or in the excretions. In addition to these general methods a number of specific tests have been developed for special vitamins which are based on particular functions of such vitamins such as for example the dark adaptation test for the determination of the presence of vitamin A and the capillary resistance test for the determination of the presence of vitamin C (or vitamin P).

17 *Hypervitaminosis*

Hypervitaminosis is according to the definition of the term the clinical syndrome which occurs when toxic amounts of a vitamin are administered.

A study of the hypervitaminoses constitutes therefore a study of the pharmacological effects of the vitamins. All vitamins in the pure state are essentially non toxic even at doses which are many times as great as those of the daily food intake. The vitamins also exert no or practically no specific pharmacological effects other than the specific vitamin action.

In experimental studies designed to determine the toxic level of vitamins, it has been found that amounts which are as high as a thousand or million times that of the daily requirement may cause the occurrence of specific syndromes of hypervitaminosis. Specificity studies of the vitamins are especially interesting from the toxicological standpoint since it has been observed that compounds which differ chemically from the vitamins only slightly may be considerably more toxic than the vitamin itself. The pharmacological study of chemical compounds usually comprises also a determination of the lethal dose. The lethal dose of most vitamins is unknown due to the fact that the vitamins are essentially non toxic.

18 Requirements

The requirements of vitamins by man and various species of animals are most important from a practical point of view. The determination of the actual amount needed proved to be a very difficult task due to the fact that the vitamin requirements vary with environment and other factors. Efforts are being made to correlate the necessary amount to either body weight or food intake according to the function of the vitamin.

Research is being directed to and national and international organizations are concerned with the problem of setting up standards for the requirements of the vitamins. The latest and most outstanding efforts of this type have precipitated the Recommended Dietary Allowances as formulated by the Food and Nutrition Board of the National Research Council. The information published by this organization is reprinted on page 613 of this monograph. These allowances fix desirable levels and are considered to be relatively liberal. They constitute norms fixed by a body of distinguished American students of nutrition available to the rest of the world for discussion and study. They furnish a guide for consideration and help to focus the aims of further experiment in the light of which further revision of the standards is to be anticipated.

The amount of vitamins which is recommended is expressed as the optimum quantity under average conditions. There was a tendency at the beginning of vitamin research to recommend as a daily dose the mini-

imum amount of vitamin which is necessary to prevent the occurrence of clinical symptoms of a deficiency disease. It was observed in the following years that the minimum amount as defined previously was not always the optimum amount which should not only prevent the occurrence of specific clinical symptoms of an avitaminosis but should also provide for certain safety factors. Generally speaking the vitamin requirements vary with age, environment and individual utilization of nutrients. Babies generally, need increased amounts; that is the vitamin requirements of babies cannot be correlated to body weight or food intake on the same basis as expressed for adults. When children grow older they still need increased amounts of vitamins but somewhat less than adults. There are some indications that aged people need increased amounts of vitamins, which seems to be due to the fact that the organism of older people utilizes food less well than adults. The influence of environment upon the vitamin requirements is especially evident for those vitamins, the average requirement for which is correlated to food intake. Thus, it has been found that approximately twice as much of vitamins B₁, B₆ and pantothenic acid are required by rats at 91° F. than at 65° F.²³

The health and general well being of a nation is dependent to a considerable extent upon the proper vitamin consumption of its people. Extensive investigations have shown that the normal food of the average man in most countries is deficient in many vitamins. It has therefore been necessary to take definite steps for providing an adequate vitamin supply to every one. This can be achieved by supplying with the necessary vitamins such foods as are generally used. Thus the Food and Nutrition Board of the National Research Council considers it good practice to fortify margarine with vitamin A and milk with vitamin D. It is also recommended and in some countries ordered to reconstitute white flour to the original content of the vitamin B complex as found for example in whole wheat grain. In the United States of America enriched flour must contain per pound 2.0-2.5 mg. of vitamin B₁, 1.2-1.5 mg. of vitamin B₂ and 16-20 mg. of nicotinic acid, and may contain 250-1000 U. S. Pharmacopoeia Units of vitamin D.²⁴

²³ C. A. Mills *Proc. Am. Physiol. Soc.* 1941 P 202

²⁴ *Federal Register, The National Archives of the United States*, July 3, 1943

**THE GROUP OF
VITAMINS A**

THE GROUP OF VITAMINS A

1 Chronology

- 1831 WACKENRODER isolated carotene from carrots
- 1904 Xerophthalmia was observed as an epidemic disease in Japan
- 1906 The empirical formula of carotene was established by WILLSTATTER
HOPKINS and STEPP (1909) discovered the indispensability of certain fat soluble substances for the growth of mice and rats
- 1913-1915 McCOLLUM and DAVIS and OSBORNE and MENDEL ascertained in differentiation from other compounds, the presence of a growth factor fat soluble A in cod liver oil and in butter
- 1919 STEENBOCK discovered the vitamin A activity of carotenoids (His results could not be verified until 1929 when H. V. FUIER showed that vitamin D must be added to the diet of animals)
- 1920 STEENBOCK recognized that vitamin A is found in the unsaponifiable parts of fish oils
- 1928-1930 ZECHMEISTER KARRER and KUHN established the constitution of carotene
- 1930 MOORE disclosed the conversion of provitamins A to vitamin A in the animal organism and storage of vitamin A in the liver after large doses of carotene
- 1931 KARRER obtained a very highly concentrated vitamin A preparation and determined the structure of the vitamin
- 1933 KARRER synthesized perhydro vitamin A
- 1937 KUHN and MORRIS announced a synthesis of vitamin A HOLMES and CORBET obtained vitamin A for the first time in crystalline form In this year the existence of a second form of this vitamin called vitamin A₂ was recognized by LEDFRER and EDISBURY and their respective co-workers

/ 2 The Group of Vitamins A

The physiological effect of vitamin A is brought about in man and in animals by a number of different naturally occurring and synthetic compounds Whereas all these substances appear to react physiologically alike they differ from each other chemically The compounds found in plants with vitamin A activity for animals, belong to the class of carotenoids with 40 carbon atoms They are apparently not used as such by the organism (at least not as vitamin A), but are converted into other substances to produce vitamin A activity These new compounds are stored to a certain extent in the animal organism in special storage organs but

have never been found in plants. Chemically, they are degradation products of the carotenoids. The most familiar of these is the one known as vitamin A which possesses the empirical formula $C_{20}H_{30}O$. All degradation products of carotenoids which occur in the animal body and react physiologically alike are called "vitamins A." The carotenes the precursors of the vitamin A, are called "provitamins A." It should be pointed out that the term provitamin is applied properly only to the specific organism for which the physiological activity has been ascertained. Rats are usually used for provitamin A studies and it is assumed, but not proved, that compounds of provitamin activity for rats also exhibit provitamin A activity for man. Nine different naturally occurring provitamins A and two vitamins A are known. However, there are many reasons for believing that more provitamins and especially, more vitamins A occur in nature.

The true physiological action and the mechanism of the vitamin A activity are essentially unknown. A few approaches have been made, however, toward solving this problem. Furthermore, a number of different diseases of man and animals are known, which are caused by a vitamin A deficiency and which can be cured by the administration of the vitamin.

PROVITAMINS A

3 Occurrence

The provitamins A occur in plants together with chlorophyll. They are generally absent from the animal organism, since they are not stored as such but are converted into vitamins A. A few exceptions to this general rule must, however, be noted. Traces of provitamins A have been found in fat deposits of animals. A special type of this class of compounds plays an important role in the visual purple of the eye and will be discussed later (see page 89). Milk and butter contain small amounts of carotenes. Egg yolk, however, contains only traces. Almost pure β carotene (and some α carotene) has been found also in the *corpus luteum*¹ and the *corpus rubrum* of cows,² in the human placenta,² the testes of bulls³ and the adrenal gland of practically all mammals.⁴ Carotenoids, the provitamin

R. Kuhn and E. Lederer *Z. physiol. Chem.* 200 248 (1931). P. Karrer and W. Schlentz *Helv. Chim. Acta* 17 8 (1934).

¹ R. Kuhn and H. Brockmann *Z. physiol. Chem.* 206 64 (1931).

² R. Netter *Bull. soc. chim. biol.* 14 1555 (1932).

³ A. A. H. van den Bergh, P. Muller and J. Broekmeyer *Biochem. Z.* 108 279 (1920). C. L. Conner *J. Biol. Chem.* 77 619 (1928). *Am. J. Path.* 4 293 (1928). H. v. Euler and E. Virgin *Biochem. Z.* 245 252 (1932). H. v. Euler, U. Gard and H. Hellström *Swensk. Kem. Tidn.* 44 191 (1932). I. Zechmeister and P. Tuxson *Z. physiol. Chem.* 231 249 (1935). O. Bailly and R. Netter *Compt. rend.* 193 961 (1931).

A character of which has not definitely been established occur also in the yellow bone marrow. The sex glands of sea urchins contain a provitamin A, echinenone which has so far not been found in plant material.

The most important sources of vitamin A for man and animals are the provitamins present in all green or yellow parts of vegetables. Carrots, apricots⁵, lettuce, cabbage and spinach are especially rich in carotenes. Tomatoes and bananas contain a lesser amount. Yellow corn contains a considerable quantity of a special provitamin A, cryptoxanthene⁶, predominantly in the form of its ester. The presence of carotenes in certain vegetable oils, especially red palm oil⁷ is technically important. The provitamins myxoxanthin and aphanin occur in algae, especially blue-green algae.

The total amount of carotenes in vegetables is rather small. Fresh carrots contain approximately 0.01% carotenes and the best source of palm oil 0.15-0.20%⁸.

The provitamins A occur in nature only to a limited extent in the free form. They are mainly bound in symplex form to proteins as has been demonstrated for carotene both in plant (carrots⁹) and in animal (serum¹⁰) materials.

4 Properties

There are nine different naturally occurring compounds known as provitamins A, namely α , β and γ carotene, cryptoxanthene, echinenone, myxoxanthin, leptotene, aphanin and aphanicin. It is quite possible that other provitamins A occur in nature which have not been discovered as yet. All these substances crystallize in deep red prisms of high melting points (above 160°). They exhibit typical absorption spectra (listed below) the positions of the maxima of which differ with the solvent used. The theory has been advanced that this shift is due to a change of the equilibrium of *cis trans* isomers¹¹.

⁵ H. Brockmann, *Z. physiol. Chem.* 216, 45 (1933).

⁶ R. Kuhn and C. Grundmann, *Be.* 67, 593 (1934).

⁷ R. Kuhn and H. Brockmann, *Z. physiol. Chem.* 200, 200 (1931).

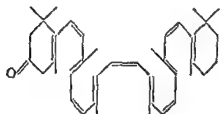
⁸ O. Ungnade, *Chem. Ztg.* 63, 9 (1939).

⁹ R. W. Lillstätt and H. H. Fischer, *Z. physiol. Chem.* 64, 47 (1910). R. Kuhn and H. J. Belg, *Be.* 73, 1080 (1940).

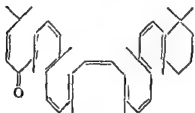
¹⁰ J. S. Pinner, *J. Biol. Chem.* 23, 261 (1911). *Carotenoids and Related Pigments*, New York, 1922, p. 108.

¹¹ E. I. Smith, B. I. Stern and P. I. Young, *Nature* 141, 1 (1938).

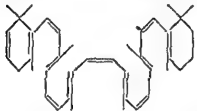
Echinenone Structure questionable¹ M p 192–193° 520 488 450 m μ (CS₂) E Lederer, *Compt rend* 201, 300 (1935) E Lederer and T Moore, *Nature*, 137, 996 (1936)



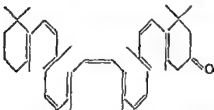
Myxoxanthin M p 168–169° 488 m μ (CS₂) 473 m μ (CHCl₃) 470 m μ (C₂H₅OH) 465 m μ (Light petroleum) I M Heilbron and B Iythgoe, *J Chem Soc* 1936, 1376



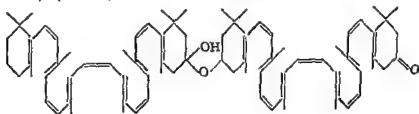
Leprotene Structure and provitamin A action questionable¹ M p 189–200° 517 479, 447 m μ (CS₂) 484, 452, 425 m μ (Benzene) C Grundman and Y Takeda *Naturwissenschaften*, 25, 27 (1937) Y Takeda and T Ohta, *Z physiol Chem*, 258, 6 (1939), 262, 168 (1939), 265, 233 (1940), 267, 171 (1941)



Aphanin Structure questionable¹ M p 180° 533 5, 494 m μ (CS₂) 504 474 m μ (CHCl₃) 505, 472 m μ (Benzene) 494 460 m μ (Petroleum) 507 5, 477 m μ (Pyridine) 491 5, 457 m μ (Methanol) J Tisher, *Z physiol Chem*, 251, 109 (1938) A Scheunert and K H Wagner, *Ibid*, 260, 272 (1939)

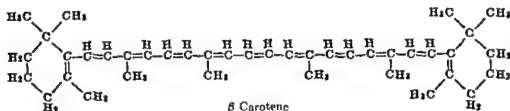


Aphanicin Structure questionable¹ M p 195° 533, 494 m μ (CS₂) 504, 474 m μ (CHCl₃) 505, 474 m μ (Benzene) 494, 462 m μ (Petro



leum) 507.5 478 $m\mu$ (Pyridine) 491.5 457 $m\mu$ (Methanol) A. Scheunert and K. H. Wagner *Z physiol Chem* 260, 272 (1939)

The aliphatic part of the molecule is written in the manner of Ruzicka in the form of open rings. This formula is not intended to indicate the true spatial arrangement of the aliphatic chain but to stress the relationship to similar compounds and to impress the memory. Textbooks commonly use the long chain formula.



Provitamins A are extremely sensitive to oxidation, autooxidation and light. They are quite stable to heat in inert atmosphere.

α Carotene is the only provitamin A with an asymmetric carbon atom and is, therefore, optically active.

The solubilities of the provitamins A are very similar. They dissolve readily in chloroform, carbon disulfide and benzene but more difficultly in petroleum ether. They are practically insoluble in alcohol. Cryptoxanthene, due to the presence of a hydroxyl group in the molecule, is somewhat soluble in alcohols. All provitamins A are soluble in fats.

5 Isolation

Crude preparations of mixtures of provitamins A are obtained by first separating the provitamins from the protein material to which they are usually bound in simplex form. This is achieved by rapid heating¹ for example to 40-60° C or by reaction with 'invert soaps' such as lauryl dimethyl benzyl ammonium bromide.¹² In the case of isolating provitamins A from vegetable oils an initial saponification with alcoholic potassium hydroxide is carried out. In either case an extraction with organic solvents such as petroleum ether follows. From the petroleum ether residue the crude mixture of carotenoids is either crystallized as such or a saponification may now be carried out in case this procedure was not employed in the beginning.

¹² R. Willstätter and H. H. Fischer *Z physiol Chem* 64, 47 (1910).

¹ R. Kuhn and H. J. Bietig *Ber* 73, 1080 (1940).

As stated mixtures of carotenoids are obtained according to these procedures. The single carotenoids are obtained by methods which will be described separately for each provitamin.

α -Carotene seldom occurs as the only carotenoid in crude provitamin A preparations. It is usually accompanied by its isomer β -carotene, and sometimes by γ -carotene. Other carotenoids may be present. The total amount of α -carotene in crude carotene preparations varies between 0% and 100%. The highest concentration of α -carotene is found in red palm oil. Almost pure α -carotene has been found in leaves of tea from Formosa.¹ The progress in separation of the α -isomer from the other carotenoids can be followed by measuring the optical activity of the preparation. α -Carotene can be isolated, for example, by fractional precipitation with xylene in the form of a dihydro- α -carotene¹² followed by regeneration of α -carotene by treatment with sodium thiosulfate.¹⁶ Fractional adsorption on aluminum oxide¹⁷ or on fuller's earth¹⁸ is a much better method. α -Carotene can be isolated quantitatively by chromatographic adsorption on calcium hydroxide or on magnesium oxide¹⁹ from a solution of a crude preparation in petroleum ether. From a mixture of the α - and β -isomers an almost quantitative separation can be achieved by a single adsorption. A red layer of β -carotene and a 100% more yellow layer of α -carotene,²⁰ are obtained.

β -Carotene is the easiest of all provitamins A to obtain in pure form. It is the only provitamin A in many plants. Its purity can easily be checked, since β -carotene is optically inactive, whereas the α -isomer has strong positive optical rotation. Small amounts of the γ -isomer can be removed by adsorption on calcium carbonate or calcium hydroxide. The separation of all provitamins A and especially of β -carotene, from xanthophylls, a yellow chloroplast pigment of green leaves, can easily and quantitatively be achieved by distribution between petroleum ether and 90% methanol.²¹ Carotenes remain almost entirely in the petroleum ether phase, while the xanthophylls pass into the methanol layer. Xanthophylls γ and δ dihydro- α -carotene.

¹ S. Yamamoto and T. Murakami, *Repts. Inst. Phys. Chem. Res. Tokyo Univ.*, 19, 157 (1932).

² K. Kuhn and F. Lederer, *Ber.*, 84, 1412 (1951).

³ K. Kuhn and F. Lederer, *Ann.*, 55, 1 (1937).

⁴ K. Kuhn and F. Lederer, *Z. physik. Chem.*, 200, 246 (1951).

⁵ K. Kuhn and H. Probstmann, *Ann.*, 550, 1 (1937).

⁶ P. Karrer, *Helv. Chim. Acta*, 18, 22 (1935).

⁷ P. Karrer and D. Walker, *Helv. Chim. Acta*, 19, 1 (1936).

⁸ K. Kuhn and F. Lederer, *Ann.*, 55, 1 (1937).

⁹ K. Kuhn and F. Lederer, *Ann.*, 55, 1 (1937).

γ -Carotene occurs only in very small quantities and is found together with β carotene. Most technical carotene preparations contain about 0.001% of the γ isomer. Occasionally, however, γ carotene occurs in larger quantities. The carotenoids of *Gonocaryum pyriforme* fruit peels consist of 50–60% γ carotene.²² It can be separated from other carotenoids by chromatographic adsorption on aluminum oxide.

Cryptoxanthene seems to occur frequently together with β carotene and other carotenoids, especially in red blossoms and fruits. Especially high in cryptoxanthene are the calyx of *Physalis alkekengi* and *Physalis franchetii* (about $\frac{1}{3}$ of the total pigment) and the pigments of yellow corn and of paprika. In all these cases, cryptoxanthene does not occur in the free form and must be saponified before isolation. Since cryptoxanthene has almost the same properties as β carotene, fractionation does not result in a separation. A number of different methods are available for separating β carotene from cryptoxanthene. Distribution between petroleum ether and 90% methanol does not bring about any separation, distribution between petroleum ether and 95% methanol, however, causes the cryptoxanthene to pass slowly into the alcohol layer. From a benzene solution of β carotene and cryptoxanthene only the latter is adsorbed on calcium carbonate. The best method for the preparation of pure cryptoxanthene is the chromatographic adsorption on aluminum oxide.

Leprotene has been isolated from bacilli, namely from *Mycobacterium phlei* and from another bacillus of unknown species found in lepra diseases. It is strongly adsorbed on aluminum oxide and can therefore be separated from the less strongly adsorbed β carotene.

Echinenone, found in the sex glands of sea urchins, occurs together with β carotene from which it can be separated by the chromatogram method.

Myxoxanthin has been found in algae of the class *Myxophyceae*. A convenient source is the fresh water species *Oscillatoria rubescens*. It has been separated from accompanying carotenoids, especially from β carotene, by chromatographic adsorption on aluminum oxide, on which it is more strongly adsorbed than β carotene.

Aphanin has been isolated from blue green algae of the *Aphanizomenon* family by chromatographic adsorption on aluminum oxide whereby it can be separated from other carotenoids, for example β carotene, which is less strongly adsorbed than aphanin.

Aphanicin occurs together with aphanin and can be separated from

²² A. Winterstein, *Z. phys. u. Chem.* 219, 249 (1933).

this and from β carotene since it is more strongly adsorbed on aluminum oxide than either of the other accompanying carotenoids

6 Chemical Constitution

The provitamins A belong chemically to a special class of polyenes, the carotenoids. They are characterized by a long aliphatic chain containing a continuous system of conjugated double bonds²³ which is responsible for their deep red color. Many naturally occurring polyenes are known but only a few of them are provitamins.

The chemical structure of all provitamins A is identical in their middle part, the symmetrical aliphatic chain of 18 carbon atoms with the continuous system of conjugated double bonds and four methyl groups constituting side chains. They differ from each other by the structure of the groups on both ends of the aliphatic chain. β Carotene has on both ends rings of the same structure as the ring of β ionone (Δ^5 1,1,5 trimethyl cyclohexene). β Carotene, therefore, is called β, β carotene. α Carotene (α, β carotene) has on one side a ring with the β ionone structure and on the other a ring with the α ionone structure (double bond in 4,5 position). γ Carotene has on one side a ring of the β ionone structure and on the other end no ring but the same number of carbon atoms as all other provitamins only in the form of an aliphatic chain (pseudo ionone structure), as in lycopene, the polyene from tomatoes. In analogy with the other carotenes γ carotene may be called β lyco β carotene. Cryptoxanthene is 3 hydroxy β, β carotene. The methyl groups, which form side chains on the molecule, are in 1,5 position to each other, corresponding to the natural principle of the structure of most terpenes, which seem to be built up from isoprene residues by dehydrogenation²⁴. Only in the middle of the chains two methyl groups are in 1,6 position, thus dividing the molecule into two symmetrical halves.²⁵

Through catalytic reduction, β -carotene takes up 11 mols of hydrogen indicating the presence of 11 double linkages²⁶. Ozonization of β carotene gives geronic acid in the same yield as obtained by ozonization of two molecules of β ionone. From α -carotene an equal amount of geronic and isogeronic acid was obtained. γ -Carotene yielded only geronic acid, in an

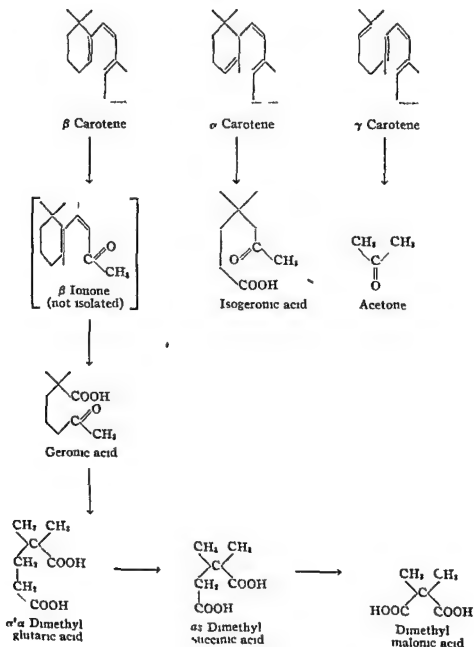
²³ R. Kuhn and A. Winterstein *Helv Chim Acta* 11 478 718 (1928). P. Karrer and H. S. Jomun *Ibid* 11 515 (1928).

²⁴ R. Willstätter and W. Mieg *Ann* 355 1 (1907). R. Kuhn and A. Winterstein *Helv Chim Acta* 11 430 (1928).

²⁵ P. Karrer and co-workers *Helv Chim Acta* 13 1087 (1930). 15 1405 (1932).

²⁶ I. Zechmeister, I. Cholnoky and V. Vrabely *Ber* 61 568 1534 (1928).

amount corresponding to the presence of one ring of the β structure. Besides the geronic acid, ozonization of γ carotene yields one molecule of acetone, split off from the aliphatic end of the molecule. γ Carotene has 12 double bonds one more than α or β carotene. Degradation of β carotene with potassium permanganate gives four mols of acetic acid.

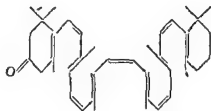


with chromic acid six mols. This proves the presence of six methyl groups in the molecule. Four of these, the ones giving acetic acid with permanganate, belong to the aliphatic chain, the other two came from methyl groups in the two rings at the ends.

Permanganate oxidation of β carotene yields α,α dimethyl glutaric acid, α,α dimethyl succinic acid and dimethyl malonic acid.

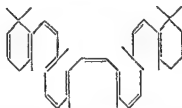
The presence of a secondary hydroxyl group in cryptoxanthene has been established by the presence of one active hydrogen atom and the formation of crystallized esters, for example, the acetate, which shows the same absorption spectrum as cryptoxanthene itself. The esters cannot be adsorbed on calcium carbonate nor can they be extracted from a petroleum ether solution by means of 95% methanol. The hydroxyl group, the exact position of which has not been determined, is assumed to occupy the carbon atom three in analogy to a number of other naturally occurring carotenoids especially lycopene.

Echinonone is a monoketone of the probable formula $C_{40}H_{58}O(=H)$. Since it shows provitamin A activity, one part of the molecule is assumed to contain the β ionone structure. It is perhaps a dehydro cryptoxanthene of the following structure



Myxoxanthin is a monoketone of the formula $C_{40}H_{54}O$ and forms an oxime. By hydrogenation 12 mols of hydrogen are taken up and 13 mols are absorbed by hydrogenation of the oxime, indicating the presence of 12 ethenoid linkages. Since myxoxanthin possesses growth promoting properties, one part of the molecule probably has the β ionone configuration. Myxoxanthin is, therefore, monocyclic and must be classified as a derivative of γ carotene. By reduction of the keto group to the alcohol group with aluminum isopropoxide, myxoxanthol is obtained which is spectroscopically identical with γ carotene.

Leprotene probably has the constitution of a dehydro β carotene. Since it contains two hydrogen atoms less than β carotene but exhibits the same absorption spectrum characteristics, the following formula is most probable



Aphanin is a monoketone of the formula $C_{40}H_{54}O$. The presence of the keto group has been established by the formation of a crystallized oxime. From the absorption spectrum of this oxime, which is the same as that of aphanin, it is concluded that the keto group is not in conjugation with the system of double bonds. The negative result of an isopropylidene determination excludes the possibility of an open chain structure at one end of the molecule. Catalytic hydrogenation indicates the presence of 12 double bonds, one of which belongs to the keto group.

Aphanicin is a dicarotenoid consisting of two molecules of aphanin linked together by an oxygen bridge. The analysis conforms with the empirical formula $C_{80}H_{106}O_2$. The presence of a keto group was ascertained by the formation of an oxime, and the molecular weight is indicated by the physiological effect of aphanicin as provitamin A.

7 Synthesis

None of the provitamins A has been synthesized as yet.

8 Industrial Methods of Preparation

The technical isolation of provitamins A from plant material is simple compared with the difficulties of manufacturing some of the other vitamins. Two different starting materials are chiefly used in industry: fresh plant material, such as carrots, nettle, lucerne, grass, etc., or vegetable oils, among which red palm oil is one of the richest sources of provitamins A.

The main difficulty in extracting carotene from fresh plant material is the presence of large amounts of water, which hinders the extraction with organic solvents. Fresh plant material must therefore be dried, which process is always attended by some loss of provitamins. The best results are obtained by a rapid, short drying process, preferably at low temperature and under vacuum. Another method is the addition of water-binding agents to the freshly ground plant material. As water-binding agents, sodium sulfate, sodium carbonate, anhydrous calcium sulfate, etc., are recommended. Another method, which, however, proved to be too ex-

pensive, is the extraction of fresh plant material with alcohol, which takes up all the water, but leaves the carotenes undissolved

The extraction of carotene from the dried material is brought about by organic solvents, such as benzene, petroleum ether, acetone, etc. These solvents dissolve, besides the carotene, all fats present. In order to obtain carotene free from other materials, and especially free from fat, a saponification of the extract must be carried out. An alternative method is the saponification of the total plant material before extraction. In most cases this latter procedure is too expensive. Since carotenes are somewhat sensitive to alkali, especially at higher temperatures (rearrangement of double bonds), the use of large excesses of alkali is carefully avoided.

The juice of plant material, for example, of carrots, is also used as starting material for carotene manufacture. The juice contains large amounts of protein materials, which adsorb the carotene when coagulated. The coagulation is carried out by the different known procedures, for example, by heating, by acids, by precipitation with lead acetate, magnesium chloride, etc. The precipitates are in turn extracted with organic solvents to isolate the carotenes.

It is not always of technical importance to prepare crystalline carotenes. Fat concentrates or dried grass and similar products are often used for food supplements.

The preparation of pure, single provitamins A is seldom carried out technically, although two different methods can be applied, namely, the precipitation of β carotene with iodine and the separation by chromatographic adsorption.

/ 9 Biogenesis

Little actual knowledge exists about the biogenesis of provitamins A although this problem has attracted widespread attention. As far as is known, only plants and certain microorganisms have the power of synthesizing provitamins A. Their constitution leads to the supposition that the building units of the provitamins are the same as those of most terpenes and polyterpenes. All these compounds appear to be built up from a simple, five carbon atom containing substance of the general formula of isoprene. Thus, all provitamins can be regarded as resulting from a condensation²⁷ together with a simultaneous dehydrogenation²⁸ of isoprene molecules. Actually, however, neither isoprene nor any other similar

²⁷ R. Willstätter and W. Mig. *Ann.* 355 1 (1917)

²⁸ R. Kuhn and A. Winterstein. *Helv. Chim. Act.* 11 427 (1928)

compound has ever been found in plants. This can be explained of course on the basis that these compounds are anabolic intermediates which are not stored as such but are used immediately for the synthesis of other compounds. Besides isoprene other compounds have been suggested as potential intermediates. Among these, β methyl crotonaldehyde,²⁹ levulinic acid³⁰ and methyl vinyl ketone are outstanding because of their obvious ability to condense with other molecules.

Without any doubt carotenoids are built up by total synthesis in plant tissues. It is questionable however whether or not the provitamins A are synthesized according to the above outlined scheme. The possibility that they might be formed by dehydrogenation of compounds of similar structure has also been discussed. Thus for example phytol³¹ or a related compound³² may serve as a starting material.

The synthesis of provitamins A appears to be catalyzed by light. Generally speaking the carotene content of plants grown in light is several times higher than the content of plants raised in the dark. Tissues of plants grown in the absence of light contain compounds which are believed to be precursors of the provitamins A. The former however are essentially colorless (perhaps saturated compounds) and differ from the provitamins in many other respects such as water solubility³⁵. On the other hand, it appears remarkable that carrot roots for example contain a more abundant amount of provitamins A than the leaves. It might be assumed of course that these provitamins were synthesized in the leaves and transported into the roots. This hypothesis is however in contradiction to the general belief that plants do not have a mechanism for the transportation of fat soluble, that is non water soluble materials. The additional hypothesis might then be considered that a water soluble precursor is synthesized in the leaves transported to the roots and there transformed (possibly by dehydration or dehydrogenation) into provitamins. On the other hand the possibility cannot be excluded that these provitamins A are totally synthesized in the roots even in the absence of light. /

10 Determination

The problem of determining quantitatively the amount of provitamins A in natural materials is quite difficult. In the analysis of plant material the

⁷ H. v. Luler and I. Klusmann, *Strahlenther. Klin. Onkol.*, **44**, 198 (1971).

¹⁰ H. L. Hodge, *H. L. C. A. m. Acta* 14, 881 (1931).

i Z čim st r n d i Chelnoky A# 465 88 (19 8)

1. K. H. Hellmuth, *Helv. Chim. Acta* 13, 1084 (1930).

R. W. H. tättler and A. Stoll: Untersuchungen über die Assimilation der A. kleinen Berlin 1918.

problem is somewhat simplified by the absence of vitamin A, which prevails in animal tissues. The methods used for the analysis of provitamins A in the presence of vitamin A are described on page 50.

✓ The only exact method for the provitamin A assay is the biological method using pure β carotene as a standard reference. This method gives average values of the total carotenoid mixture expressed as β carotene equivalent (see page 82). ✓

Approximate provitamin A values can be obtained by either physical or chemical methods. They involve a separation of the provitamins A from other carotenoids present especially xanthophyll (lutein) and other phyto xanthins. Such a separation is based on the fact that α , β and γ carotenes are hydrocarbons and the phyto xanthins are oxygenated carotene derivatives, that is, alcohols and ketones. They show different solubility characteristics and can therefore be separated to a certain extent. These separations do not bring about a separation of provitamins A which contain hydroxyl or keto groups from non provitamin A carotenoids with such functional groups. Fortunately the average provitamin A analysis in foodstuffs can disregard all provitamins A other than the carotenes proper, since they are found only infrequently.

The actual assay procedure involves, first the separation of the provitamin A from the protein to which it is bound in animal and plant materials. This is accomplished either by heating to 40–60° C or by the addition of invert soaps such as lauryl dimethyl benzyl ammonium bromide³¹. This is followed by an extraction from the natural source, either before or after a saponification. The alcohols, ethers, petroleum ether, hexane, acetone, pyridine and mixtures, such as petroleum ether and methanol or petroleum ether and acetone, have been recommended as solvents. The saponification is carried out with potassium hydroxide in alcohol preferably at room temperature although the application of heat is recommended when entire tissues are to be saponified. Saponification with aqueous potash has also been advocated. The main object is to bring the pigments in the saponified form into a solution of petroleum ether. This solution is then washed with dilute alkali and water to effect a separation from the chlorophyll. The actual separation of the carotenoids is then accomplished by shaking the petroleum ether solution with 92% methanol (by volume). The hydrocarbons remain in the petroleum ether layer while the oxygenated xanthophylls go into the methanol phase³. After repeated extractions, the pe-

³¹ R. Kuhn and H. J. Brielig, *Ber.* 73, 1080 (1940).

R. Willstätter and A. Stoll, *Untersuchungen über Chlorophyll*, Berlin, 1913.

petroleum ether solution is used for direct determinations³⁶ ✓ Thus the total amount of carotenes can be determined spectrophotometrically and is usually expressed in terms of β carotene. Determinations are usually made of only one wave length using the 450 m μ band of β carotene for which $E_{1\%}^{1\text{cm}} = 2500$ (in petroleum ether). It has also been suggested that the provitamins A be determined colorimetrically by comparison with the color of standard dye solutions for example, of azobenzene of known concentrations³⁷ or of a 0.1% solution of KCr_2O_7 ^{38, 39} ✓

Another method is to develop a blue color according to the method of Carr and Price (see page 78) and to determine the color by spectrophotometric methods ✓

Qualitative estimations for the presence of individual provitamins A can be made by using the chromatographic adsorption technique for the separation from other carotenoids. The methods used are the same as described for the preparation of the individual provitamins A. The principle of preferential adsorption has also been developed for the quantitative estimation of carotenes. Thus a specially prepared magnesium carbonate has been developed to absorb xanthophyll but not carotene^{40, 41} and to absorb lycopene but not carotene^{42, 43}.

CONVERSION OF PROVITAMINS A INTO VITAMINS A

Most substances ingested by human beings and by animals which bring about vitamin A action are not identical with the vitamins A found in animal organisms. They are provitamins A. In 1919 Steenbock⁴⁴ found that A avitaminosis in rats can be cured with fresh green plant materials and that the healing action corresponded to the amount of carotene present. This result could not be repeated until it was demonstrated ten years later⁴⁵ that the avitaminotic animals needed also vitamin D to regain health. It was furthermore discovered that the vitamin A content of the liver of

³⁶ Tentative method of the Association of Official Agricultural Chemists for the determination of carotene. *J. Assoc. Official Ag. Chem.* 22: 79 (1939).

³⁷ P. Karrer and K. Schöpp. *Helv. Chim. Acta* 15: 743 (1932). R. Kulsh and H. Brockmann. *Z. physiol. Chem.* 206: 41 (1932).

³⁸ H. R. Guibert. *Ind. Eng. Chem. Anal. Ed.* 6: 457 (1934).

³⁹ V. E. Munsey. *J. Assoc. Official Ag. Chem.* 22: 861 (1933).

⁴⁰ G. S. Frap and A. R. Kemmerer. *Ibid.* 22: 190 (1939).

⁴¹ G. S. Frap, A. R. Kemmerer and S. M. Greenberg. *Ibid.* 23: 4 (1940).

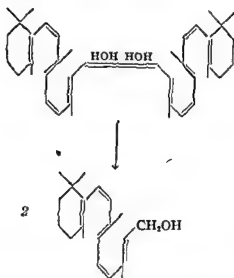
⁴² G. S. Frap, A. R. Kemmerer and S. M. Greenberg. *Ibid.* 23: 6 (1940).

⁴³ H. Steenbock. *Science* 50: 757 (1919). H. Steenbock and P. W. Boutwell. *J. Biol. Chem.* 41: 81 (1920). H. Steenbock, M. T. Self, I. M. Nelson and M. V. B. *Ibid.* 46: 3 (1921). H. Steenbock and I. C. Crooks. *Ibid.* 51: 63 (1924).

⁴⁴ B. V. Fidler, H. V. Fidler and P. Karrer. *Helv. Chim. Acta* 12: 78 (1929). H. Fidler and Demole. *I. Karrer and O. Walker. Ibid.* 13: 1078 (1930).

rats decreased⁴⁵ rapidly and finally disappeared completely when the animals were kept on a vitamin A free diet. After the addition of carotene to the diet, the vitamin A content of the liver increased again rapidly.

At the same time, vitamin A was isolated from liver oils and its formula was established.⁴⁶ Vitamin A contains just half as many carbon atoms as carotene. Theoretically, two mols of vitamin A must split out two mols of water to become β carotene.



The hypothesis was then advanced that β carotene is split in the organism with the addition of two molecules of water to yield two molecules of vitamin A. This theory was strongly supported by the difference in the quantitative physiological efficacy of α and β carotene. There exist however trustworthy observations⁴⁷ which indicate that even under optimum conditions an unsymmetrical fission of provitamin A occurs which in the case of β carotene yields a maximum of only one mol of vitamin A plus other decomposition products. This is in agreement with the observed potency of provitamin A in many cases (see page 75).

The mechanism of the conversion of provitamins A to vitamins A is not known. It is assumed that the conversion is effected by an enzyme called carotenase. It is not known with certainty in which organ the conversion takes place, but much evidence exists that it is in the liver.⁴⁸ Furthermore, the pancreas appears to be involved since in human beings with

⁴⁵ T. Moore, *Biochem. J.* 24, 61 (1930).

⁴⁶ P. Karrer, R. Morf, and K. Schöpp, *Helv. Chim. Acta* 14, 1036, 1431 (1931).

⁴⁷ W. F. Underhill and E. H. Coward, *Biochem. J.* 33, 689 (1939). H. v. I. der. P. Karrer and A. Zubrys, *Helv. Chim. Acta* 17, 24 (1934).

⁴⁸ H. S. Olcott and D. C. McCam, *J. Biol. Chem.* 94, 185 (1931). B. Ahmad, *Biochem. J.* 25, 1105 (1931). J. I. Rea and J. C. Drummond, *Z. Vitaminforsch.* 1, 177 (1937).

tinal tract contains smaller amounts. Considerable quantities are present in the retina and the corpora lutea, small amounts in lungs and kidney.⁶⁹ Vitamin A is present also in egg yolks (one hen egg contains about 20 U S Pharmacopoeia Units),⁷⁰ milk (3 U S Pharmacopoeia Units per gram)⁷¹ and milk products (butter, 50 U S Pharmacopoeia Units per gram). Colostrum of man and of cows is from two to ten times richer in vitamin A than the milk. Stored fats contain small amounts of vitamin A, especially during pregnancy.

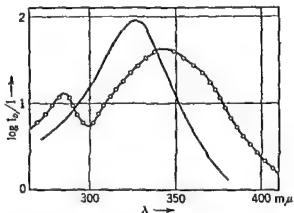


Fig. 3—Absorption spectra of vitamin A (—) and of vitamin A₂ (O—O—O) (A. E. Gillam, I. M. Heilbron, W. E. Jones and E. Lederer)

vitamin A occurs both as free alcohol and predominantly in esterified form in the animal organism.⁷² Vitamin A palmitate has been isolated from the liver oil of *Stereolepis ishnagi*⁷³ and from cod liver oil⁷⁴ in the form of an addition product with maleic anhydride. The free vitamin A has been separated⁷⁵ from its esters by molecular distillation. By the use of this method it has also been shown that different esters are present in cod liver oil and cod liver oil.

Biochem J 25 7 (1931)

and E. Klusmann *Physchem Z* 219 215 (1933)

and I. M. Heilbron

Biochem J 24 880 (1930)

T. Moore *Biochem J* 26 1

and E. I. Gillam

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Reseach (Tokyo) 26 87 (1935) K. Kawakami *ibid*

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12 Occurrence

Vitamin A occurs only in the animal organism, it has not been found in plants.

The most important source of vitamin A is liver oils because the organism stores most of the excess vitamin A in the liver. The amount of vitamin A in the liver varies greatly, according to the type of food consumed, general living conditions, season etc. Fish liver oils from cod, halibut, tuna, etc., contain vitamin A in fair amounts. Especially rich in vitamin A are the liver oils from *Hippoglossus hippoglossus*, *Scombresox saurus*, *Rhombus maximus* and from the Japanese fish, *Stereolepis ishnagi* which contain from 200 to 2000 times as much vitamin A as the common cod liver oil.

TABLE I
DISTRIBUTION OF VITAMIN A IN VARIOUS FISH LIVER OILS

Source of oil	Zoological name	Potency I U per gram
Haddock	<i>Gadus aeglefinus</i>	65
Cod	<i>Gadus morrhua</i>	600
Striped bass	<i>Morone saxatilis</i>	4 500
Jack smelt	<i>Atherinopsis californiensis</i>	10 000
Albacore	<i>Scomber alalunga</i>	18 000
Grouper	<i>Epinephelus morio</i>	25 000
Boston mackerel	<i>Scomber scombrus</i>	30 000
Barracuda	<i>Sphyræna argentea</i>	40 000
Shipjack tuna	<i>Katsuwonus pelamis</i>	40 000
Yellowtail	<i>Seriola dorsalis</i>	50 000
White sea bass	<i>Cynoscion nobilis</i>	50 000
Red snapper	<i>Lutjanus campechanus</i>	60 000
Totuava	<i>Erisicton macdonaldi</i>	60 000
Bluefin tuna	<i>Thunnus thynnus</i>	60 000
Yellowfin tuna	<i>Neothunnus macropterus</i>	70 000
Pacific mackerel	<i>Pneumatophorus diego</i>	80 000
Bonito	<i>Sarda chiliensis</i>	120 000
Cabrilla punta	<i>Epinephelus analogus</i>	170 000
Swordfish	<i>Xiphus gladius</i>	250 000
Ishnagi	<i>Stereolepis ishnagi</i>	300 000
Black sea bass	<i>Stereolepis gigas</i>	600 000

Besides the liver, other organs contain some vitamin A the most important of which are the pyloric ceca of a number of fish species. These contain an amount equal to that found in the liver⁶⁸. The rest of the intestines

⁶⁸ J. R. Edmury, R. A. Morton, C. W. Smykns and J. A. Jovern, *J. Biol. Chem.* 32: 118 (1958)

final tract contains smaller amounts. Considerable quantities are present in the retina and the corpora lutea, small amounts in lungs and kidney.⁶⁹ Vitamin A is present also in egg yolks (one hen egg contains about 20 U S Pharmacopoeia Units),⁷⁰ milk (3 U S Pharmacopoeia Units per gram)⁷¹ and milk products (butter, 50 U S Pharmacopoeia Units per gram). Colostrum of man and of cows is from two to ten times richer in vitamin A than the milk. Stored fats contain small amounts of vitamin A especially during pregnancy.

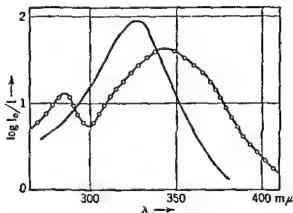


Fig. 3—Absorption spectra of vitamin A (—) and of vitamin A₂ (o-o-o) (A. E. Gillam, I. M. Heilbron, W. F. Jones and F. Lederer)

Vitamin A occurs both as free alcohol and predominantly in esterified form in the animal organism.⁷² Vitamin A palmitate has been isolated from the liver oil of *Stereolepis tshnagi*⁷³ and from cod liver oil⁷⁴ in the form of an addition product with maleic anhydride. The free vitamin A has been separated⁷⁵ from its esters by molecular distillation. By the use of the same method it has also been shown that different esters are present in halibut liver oil and cod liver oil.

⁶⁹ T. Moore, *Biochem. J.* 25, 25 (1931).

⁷⁰ H. v. Euler and E. Klusmann, *Biochem. Z.* 219, 215 (1933).

⁷¹ R. A. Morton and I. M. Heilbron, *Biochem. J.* 24, 860 (1930); T. Moore, *Biochem. J.* 26, 1 (1932).

⁷² A. L. Bacharach and E. I. Smith, *Quart. J. Pharm.* 1, 539 (1932); *Ret. Compt. and soc. Biol.* 120, 577 (1935).

⁷³ Hamano, *Sci. Papers in Phys. Chem. Research (Tokyo)* 26, 87 (1935); K. Kawakami, *Ibid.* 26, 7 (1935).

⁷⁴ K. C. D. Hickman, *Ind. Eng. Chem.* 29, 968, 1107 (1937).

⁷⁵ A. O. Fischer, *J. Biol. Chem.* 125, 475 (1938).

13 Properties

Vitamin A crystallizes from methanol in pale yellow plates, containing solvent of crystallization and melting at 8°C ⁷⁷. A purer form of vitamin A crystals, melting at $63\text{--}64^{\circ}\text{C}$ is obtained from ethyl formate or from propylene glycol⁷⁶. Vitamin A was found to distill at $137\text{--}138^{\circ}\text{C}$ at 10^{-4} mm⁷⁸ but later workers found the material to evaporate at $120\text{--}125^{\circ}\text{C}$ at 5×10^{-3} mm⁷⁹. The natural vitamin A esters distill from $200\text{--}240^{\circ}\text{C}$ at 10^{-1} mm. The absorption spectrum of vitamin A in the ultraviolet shows a sharp band at the wave length $328\text{ m}\mu$ (Fig. 3) with an extinction coefficient $E_{1\%}^{1\text{cm}} = 1725$ ⁷⁷. The biological activity is 4 500 000 International Units per gram⁸⁰.

Vitamin A is sensitive to oxidation and autoxidizes readily. It is quite heat stable in inert atmosphere and is readily stabilized in solution in oil. The esters of vitamin A are more stable than the free substance. Vitamin A is destroyed by ultraviolet light and is optically inactive. Vitamin A crystals appear to be isotropic. Vitamin A is soluble in most organic solvents but is insoluble in water. It has, therefore, been classified as a member of the fat soluble vitamins.

14 Isolation

Vitamin A can be isolated from the unsaponifiable parts of animal fats, especially liver oils. Three different methods have been used for the isolation, namely, chromatographic adsorption, vacuum distillation and fractional crystallization at low temperatures.

The use of the chromatographic adsorption method⁸¹ brought about the first preparation of almost pure material. The liver oils of *Hippoglossus* and of the mackerel *Scombrex saurus* were saponified and the unsaponifiable part freed from sterols by cooling to -70°C . The non-crystallized material possesses the vitamin A activity. By adsorption on aluminum oxide followed by differential adsorption on calcium hydroxide a concentrate was obtained, which by repeated adsorption could not be further

⁷⁶ J. G. Baxter and C. D. Robeson *Science* 92 206 (1940)

⁷⁷ H. N. Holmes and R. E. Corbet *J. Am. Chem. Soc.* 59 2042 (1937)

⁷⁸ J. M. Heibron, R. N. Heslop, R. A. Morton, E. T. Webster, J. L. Rea and J. C. Drummond *Biochem. J.* 26 1178 (1932); F. H. Carr and W. J. Jewell *Nature* 131 92 (1933)

⁷⁹ K. C. D. Hickman *Ind. Eng. Chem.* 29 968 1107 (1937)

⁸⁰ J. G. Baxter, P. L. Harris, K. C. D. Hickman and C. D. Robeson *J. Biol. Chem.* 141 991 (1941)

⁸¹ P. Karrer, R. Morf and K. Schöpp *Helv. Chim. Acta* 14 1036 1431 (1931)

purified⁸. It consisted of a viscous yellow oil which was used for the successful determination of the chemical structure.

The disadvantage of this method lies in the fact that due to the sensitivity of the vitamin A molecule partial destruction occurs during the adsorption procedure. Rearrangements of the double bonds and slight oxidation might be the chief reasons for the change of the compound.

By the use of the vacuum distillation process vitamin A concentrates have been obtained^{83, 84, 85, 86} of the same properties as obtained by the use of the chromatographic adsorption method. A decided improvement is the use of the short path high vacuum distillation which has resulted in the isolation of vitamin A and its esters in relatively pure form and has enabled the determination of exact boiling points and distillation characteristics. This has been accomplished by the use of the cyclic molecular still and certain adjuncts such as constant yield oils and pilot dyes. The saponified vitamin concentrate is dissolved in a mixture of neutral residue oil and constant yield oil and is distilled in stepwise fashion over long ranges of temperatures. The concentration of vitamin A in the distillates is plotted against temperature and results in an 'elimination curve' with a distillation maximum on the temperature axis. Using this method vitamin A is shown to distill at 123° as compared with celanthrene red distilling at 125° C.⁸⁷

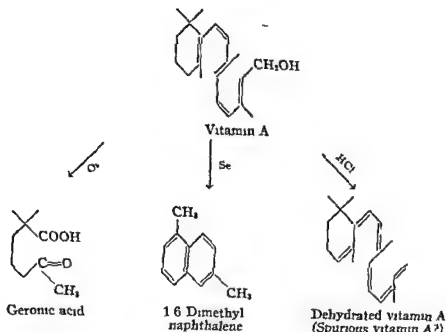
Vitamin A was finally crystallized⁸⁸ by the use of fractional freezing and cold filtration after the isolation of the unsaponifiable material. Vitamin A crystals are best obtained by crystallization from ethyl formate or from propylene oxide.⁸⁹ From methanol vitamin A crystallizes with solvent of crystallization. Pure vitamin A melts at 63.64° C.⁸³

15 Chemical Constitution

The constitution of vitamin A has been determined mainly by Karrer who suggested the following structure,

- ⁸ P. Karrer and R. Morf *Helv Chim Acta* 16 6 (1933)
- ¹ M. Heilbron, R. N. Heslop, R. A. Morton, J. I. Webster, J. I. Rea and J. C. Drummond *Biochem J* 26 1178 (1932)
- ⁸³ F. H. Carr and W. J. Jewell *Nature* 131 52 (1933)
- ⁸⁴ J. C. Drummond, H. J. Channon and K. H. Coward *Bi. chem J* 19 1047 (1925)
- ⁸⁵ Vitamin A prepared according to this method was used in 1935 to set up a provision standard
- ⁸⁶ E. M. Hume and H. Chick *Med. Research Council Rept. IV The Standardisation and Estimation of Vitamin A* 193
- ⁸⁷ K. C. D. Hickman *Ind. Eng. Chem.* 29 968 1107 (1937) D. H. Killeffer *Ibid.* 29 366 (1937)
- ⁸⁸ N. D. Embree *Ibid.* 29 915 (1937) J. G. Baxter, F. I. Gray and O. A. Tinker *Ibid.* 29 1112 (1937)
- ⁸⁹ H. N. Holmes and R. F. Corbet *J. Am. Chem. Soc.* 59 2042 (1937)
- ⁹⁰ J. G. Baxter and C. D. Robeson *Science* 92 202 (1940)

VITAMIN A



The empirical formula is $\text{C}_{20}\text{H}_{30}\text{O}$. Various crystallized esters for example the beta naphthoate m p 76°C the anthraquinone β carboxylic acid ester m p 126°C ⁹⁰ the acetate m p $56-58^\circ\text{C}$, the palmitate m p $26-28^\circ\text{C}$ and the disuccinate m p $73-75^\circ\text{C}$, have been prepared and indicate the alcoholic function of the oxygen. Molecular weight determinations proved to be extremely difficult to carry out due to rapid changes of the vitamin in solution. The best experimental values obtained show an average molecular weight of 294 but it seems probable that the correct weight must be a little less⁹¹ (Karrer's formula calls for 286).

Oxidation of vitamin A with ozone yields geronic acid which proves the presence of the β ionone ring. The methyl groups in the side chain are converted into acetic acid by oxidation with permanganate in analogy to the results obtained by the oxidation of the provitamins A. Dehydrogenation of vitamin A with selenium forms 1,6 dimethyl naphthalene by closing a second ring and splitting off two methyl groups and part of the side chain⁹². By the action of mineral acids on vitamin A a transformation occurs, which is indicated by a change of the absorption spectrum. It has been suggested⁹³ that under the influence of acids a second ring is formed

⁹⁰ S. Hamano *Sci. Papers Inst. Phys. Chem. Research (Tokyo)* 28: 69 (1931); 32: 44 (1937). T. H. M. *Ad. Biochem. J.* 33: 584 (1939).

⁹¹ H. N. Brown, J. Orvathoff and J. J. Wolf *Biochem. J.* 25: 430 (1931). H. N. Holmes and R. I. Corbet *J. Am. Chem. Soc.* 59: 2042 (1937).

⁹² I. M. Heilbron, R. A. Morton and E. T. Webster *Bi. chem. J.* 26: 1114 (1932).

⁹³ J. R. L. D. bury, A. F. Callam, I. M. Heilbron and R. A. Morton *Ibid.* 26: 1161 (1932).

It is more plausible that a dehydration may take place. A compound apparently identical with dehydrated vitamin A and often referred to as spurious vitamin A has also been found to be present as a normal constituent of fish liver oils. (The absorption maxima occur at 350, 368 and 379 m μ .⁹⁴)

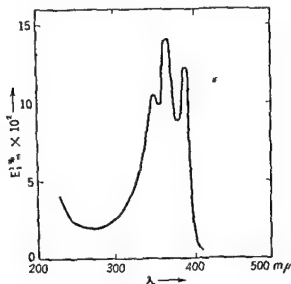
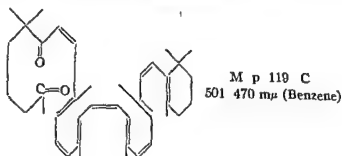


Fig. 4—Absorption spectrum of anhydro vitamin A (N. D. Embree)

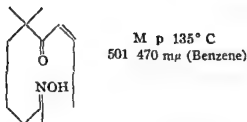
Vitamin A contains a continuous series of five conjugated double bonds. Maleic and citraconic anhydrides form addition products of unknown constitution.⁹⁵ By catalytic hydrogenation of vitamin A five mols of hydrogen are absorbed. The reaction product perhydro vitamin A has been prepared synthetically according to the following scheme.⁹⁶ β Ionone and bromo ethyl acetate were condensed with zinc according to Reformatsky forming β ionylidene ethyl acetate (II). By catalytic hydrogenation the double bonds were saturated and by reduction with sodium and alcohol according to Bouvaut Blanc the ester group was converted into an alcohol.

D C Castl A L Gll m I M H lbron and H W Thompson *B chem J* 28 170 (1934)
I M Helbron R N H lop R A Morton J L R a nd J C Dr mmmond *Ibid* 26 11 8 (193)
H Pritch rd H Wilkinson J R Edisbury and R A Mo ton *Ib d* 31 58 (1937) N D I mbree
J Biol Chem 128 187 (1933)
* H n ano Yc *Fap v In s I hys Chem Res ch (Toky)* 25 536 38 (1934) 26 8* (1935)
K Kaw k m *Ib d* 26 77 (193) / Nak mya *Ib d* 27 4 (193)
I K arter and R Morf *Helv Chim Acta* 16 557 62 (1933)

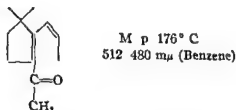
in a daily dose of 5 γ . Carotene iodide is also active in a daily dose of 40 γ probably due to a regeneration of carotene ¹⁴¹



(I)
 β Semi-carotenone



(II)
 β Semi-carotenone monoxime



(III)
 β Dehydro semi carotenone

Some of the degradation products of β carotene are biologically active whereas the corresponding derivatives of α carotene appear to be inactive. By mild oxidation of β carotene with permanganate a series of aldehydes was obtained by fission of various double bonds. Thus, the main reaction product, β apo 2 carotinal¹⁴² (formula (II) on page 77) is obtained by an oxidation of the double bond in the aliphatic chain of the molecule adjacent to one ring. This compound as well as its oxime proved to be active in a daily dosage of 5 γ .¹⁴³ The vitamin A produced from β apo 2 carotinal in rats resembles in all characteristics the vitamin A or avero phthol.¹⁴⁴ In other words this degradation product of β carotene is apparently not a vitamin A but a provitamin A. By reduction with aluminum isopropylate, β apo 2 carotinal can be converted into the corresponding alcohol β apo 2 carotinol, which compound probably also is a provitamin A. By condensation of β apo 2 carotinal with ethyl magnesium bromide, a tertiary alcohol of the formula (III) is produced, which is also active.¹⁴⁴

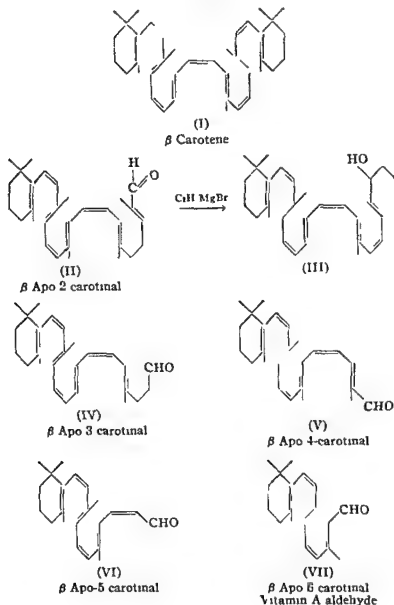
¹⁴¹ P. Karrer and M. Rydholm *Ber* 62 2445 (1929). T. Moore *Lancet* 1929 II 219.

¹⁴² P. Karrer and U. Solmssen *Helv. Chim. Acta* 20 68° (1937).

¹⁴³ H. v. Euler, P. Karrer and U. Solmssen *Ibid.* 21 211 (1938).

¹⁴⁴ H. v. Euler, G. Günther, M. Malmberg and P. Karrer *Ibid.* 21 1619 (1938).

¹⁴⁵ P. Karrer, A. Ruegger and A. Geiger *Ibid.* 21 1171 (1938). H. v. Euler, G. Günther, M. Malmberg and P. Karrer *Ibid.* 21 1619 (1938).



The next lower homolog of β apo 2 carotinal is β apo 3 carotinal (for mula (IV)) which is present in the oxidation products in small quantities, but which has not been isolated. β Apo-4 carotinal has been obtained however in the form of its crystallized oxime which is biologically active. The corresponding alcohol has also been prepared. The next lower homolog would be β apo 5 carotinal the alcohol of which has the formula which has tentatively been assigned to vitamin A₂. This compound has not yet

been isolated from the oxidation products of carotene, although lower homologs are present. The absorption spectra of the series of 'homolog' alcohols of axerophthol indicate a constant shift of the maxima toward higher wave lengths with an increasing number of double bonds present (see Fig 5). From these data it appears questionable that vitamin A₂ has the formula of a β apo 5 carotinol

20 Determination

(a) Chemical Methods

A great number of different color reactions have been proposed for the determination of vitamin A. These color tests are based on three different types of reactions: reaction with phenols, with acids, and with inorganic chlorides, which in aqueous solution show an acidic reaction.

The method of Carr and Price¹⁴⁸ is the only one in general use. A 20-25% solution of antimony trichloride in chloroform is added to a solution of vitamin A in chloroform. To insure greatest possible accuracy in the determination the antimony trichloride reagent should be free of any traces of ferric chloride.¹⁴⁶ A blue color develops which shows its maximum after 10 seconds and then immediately begins to fade. The color is measured in a colorimeter for example, in the Lovibond tintometer (visual or photoelectric¹⁴⁷ observation), or by spectrophotometric analysis. The values obtained may be checked against standardized solutions of copper sulfate¹⁴⁹ and cobalt nitrate or against certain color filters. The value of a 20% solution of the vitamin containing material in chloroform is called a

Lovibond Unit. For international use a 2% solution is recommended. The values obtained by this latter method are called "C. I. O." (cod liver oil) Units. Various other manners of expressing the color value have been recommended for example the Carr Price value,¹⁴⁸ the Moore¹⁵⁰ blue units and the Dann Evelyn¹⁵¹ L 620 m μ value. A noteworthy variation is the Anderson Nightingale¹⁵ dilution test which consists in diluting the material to be analyzed for its vitamin A content with chloroform until the blue color is just visible. (For comparison of different units see also page 83.)

¹ J. R. Edisbury *Analyst* 65 484 (1940)

¹⁴ R. L. McFarlan, J. W. Reddie and E. C. Merrill *Ind. Eng. Chem. Anal. Ed.* 9 34 (1937)

¹⁵ F. H. Carr and F. A. Price *Biochem. J.* 20 498 (1926)

¹⁶ H. Brockmann and M. I. Tecklenburg *Z. physiol. Chem.* 221 117 (1932)

¹⁴⁸ T. Moore *Biochem. J.* 23 1267 (1929); 31 155 (1937)

¹⁴⁹ W. J. Dann and K. A. Evelyn *Ibid.* 32 1008 (1938)

¹⁵¹ A. Andersen and I. Nightingale *J. Soc. Chem. Ind.* 48 139T (1929)

The values obtained with the Carr Price method for vitamin A from liver oils are in fairly good agreement with the results of biological tests. Crude liver oils contain substances which interfere with the color reaction¹⁵⁴ and should therefore be saponified under anaerobic conditions and in the absence of sunlight before testing. Some naturally occurring acids decrease the value¹⁵⁵. Some carotenoids for example cyclized vitamin A increase the value. It is possible, however, to differentiate by the use of selective filters between the colors produced by vitamin A and by carotenes by measuring the absorption, the maximum being at 620 m μ for vitamin A and at 590 m μ for carotene. The maximum for vitamin A₂ lies at 693 m μ . Impurities and mixtures of vitamins A and carotenes may change the wavelength of the maximum of absorption¹⁵⁶. Visually the colors produced by carotene and vitamin A can be differentiated since the blue obtained from carotene persists without fading while the blue from vitamin A fades in two to five minutes. A mixture is indicated when the color fades rapidly at first but remains constant later on. A red color is caused by the presence of sterols¹⁵³. The Carr Price color reaction cannot be used for the determination of biologically active material in synthetic vitamin A preparations.

Antimony trichloride causes a change in the chemical structure of the carotenoids the nature of which is unknown. The reaction product is biologically inactive¹⁵⁷.

A number of modifications of the Carr Price test have been proposed for example the addition of a 0.5% pyrocatechol¹⁵⁸ the addition of arsenic trichloride and hydroquinone¹⁵⁹ determination at low temperature¹⁶⁰ determination of the blue value minus the yellow value¹⁶¹ etc.^{162, 163}

Y. Raoul and P. Meunier *J. pharmacol.* 29, 112 (1933).

E. R. Norris and A. E. Church *J. Biol. Chem.* 85, 477 (1930); 87, 131 (1930); 89, 421 (1930).
F. I. Smith and V. Hazley *Biochem. J.* 24, 1942 (1930); K. H. Coward, F. J. Dyer, R. A. Morton and J. H. Gaddum *Ibid.* 25, 1107 (1931); R. S. Morgan *Ibid.* 26, 1144 (1932).

¹⁵⁴ A. Emmerie *Vain.* 131, 364 (1933); *Acta Biotheoretica et Land. Physiol. Pharmacol. Microbiol.* 2, 158 (1933); *Proc. Roy. Soc. Amsterdam* 35, 1347 (1932).

¹⁵⁵ H. v. Euler, P. Karrer, E. Klusmann and R. Morf *Helv. Chim. Acta* 15, 50 (1932); H. Brockmann and M. I. Tecklenburg *Z. physiol. Chem.* 221, 117 (1933); I. M. Heilbron, A. F. Gillam and R. A. Morton *Biochem. J.* 25, 1352 (1931); M. v. Eekelen, A. Emmerie, H. W. Jullius and K. I. Wolf *Acta Biotheoretica et Land. Physiol. Pharmacol. Microbiol.* 1, 8 (1931).

¹⁵⁶ A. L. Gillam, I. M. Heilbron, R. A. Morton and J. C. Drummond *Biochem. J.* 26, 1174 (1932).

¹⁵⁷ J. Rosenthal and J. Frédy *Ibid.* 28, 41 (1934).

¹⁵⁸ G. Gutzit *Arch. sci. phys. nat.* 9, 155 (1927).

¹⁵⁹ E. R. Norris and co-workers *J. Biol. Chem.* 85, 47 (1929); 89, 421 (1930); *J. Nutrition* 5, 49 (1932).

¹⁶⁰ R. S. Morgan *Biochem. J.* 26, 377 (1932).

¹⁶¹ F. I. Smith and V. Hazley *Ibid.* 24, 1942 (1930); 27, 17 (1933).

¹⁶² T. Moore *Ibid.* 24, 892 (1930).

The Carr-Price method uses the principle of developing a color of vitamin A with an inorganic chloride in anhydrous solvents. The same principle is used in tests with aluminum chloride, arsenic chloride,¹⁶⁴ ferric chloride, or stannic chloride.¹⁶⁵ The principle of using acids to produce a measurable color with vitamin A has been recommended. Sulfuric acid, phosphoric acid,¹⁶⁶ chloric acid,¹⁶⁷ molybdenum phosphotungstic acid,¹⁶⁸ and trichloroacetic acid¹⁶⁹ are used either alone or in combination with the phenol principle, to produce colors.¹⁶⁷⁻¹⁶⁹ The latter principle is also used alone, for example, by means of pyrogallol.¹⁶⁹⁻¹⁷⁰

The phenol principle has also been applied in conjunction with inorganic chlorides.¹⁷¹

All these methods are equally as accurate or less so than the Carr-Price reaction. None of these has been adopted for the determination of vitamin A by any official organization.

Carotene in the presence of vitamin A can be determined by either chromatographic adsorption¹⁷ or by colorimetric measurements¹⁷² of the color developed by the Carr-Price reaction (see above) or by direct spectroscopic examination.

The amount of free vitamin A in mixture with vitamin A esters can be estimated by the behavior of the free vitamin toward methanol. From an oily solution containing both the free and the esterified vitamin A, about 40–60% of the free vitamin, but no esters, is extracted with methanol.¹⁷⁴ Molecular distillation is another method for the separation of the two forms.¹⁷⁵

(b) Physical Methods

The extinction coefficient of the characteristic absorption band at 325–328 m μ can be used for the quantitative determination of vitamin A.¹⁷⁶

¹⁶ O. Rosenheim and J. C. Drummond, *Biochem J.* 19, 753 (1925).

¹⁶⁵ F. H. Carr and E. A. Price, *Ibid.* 20, 497 (1926).

¹⁶⁶ F. Kobayashi and K. Yamamoto, *J. Soc. Chem. Ind. Japan* 27, 1060 (1924).

¹⁶⁷ A. E. Pacini and M. H. Taras, *J. Am. Pharm. Assoc.* 26, 721 (1937).

¹⁶⁸ N. Bezsonoff, *Bull. soc. chim. biol.* 11, 294 (1929).

¹⁶⁹ W. R. Fearon, *Biochem J.* 19, 888 (1925).

¹⁷⁰ T. Moore, *Lancet* II, 219 (1929). S. G. Willmott and F. Wokes, *Ibid.* II, 8 (1927). O. Rosenheim and T. A. Webster, *Ibid.* II, 806 (1926). *Biochem J.* 20, 1342 (1926).

¹⁷¹ J. Rosenthal and J. Erdélyi, *Biochem Z.* 271, 414 (1934).

¹⁷² P. Karrer and K. Schöpp, *Helv. Chim. Acta* 15, 745 (1932).

¹⁷³ R. Kuhn and H. Brockmann, *Z. physiol. Chem.* 206, 41 (1931).

¹⁷⁴ V. Ritsch, *G. P.* 636, 727.

¹⁷⁵ K. C. D. Hickman, *Ind. Eng. Chem.* 29, 968, 1107 (1937).

¹⁷⁶ R. A. Morton, *Practical Aspects of Absorption Spectrophotometry*. In title of Chemistry, 1938.
I. M. Heibron and co-workers, *Biochem J.* 22, 95* (1928). 24, 870 (1930). A. Chevallier and P. Chabre, *Ibid.* 27, 298 (1933). K. H. Coward, F. J. Dyer, R. A. Morton and J. H. Gaddum, *Ibid.* 25, 1102 (1931). A. Chevallier and P. Doubouloz, *Bull. soc. chim. biol.* 18, 190 (1936).

Care must be taken in choosing the solvent that is, either cyclohexane, ethyl or isopropyl alcohol has been recommended. The absorption bands vary somewhat both in position and in magnitude with change of solvents. As much as 10% increase (in ether) or 10% decrease (in chloroform) has been observed in comparison with the extinction coefficient of the same sample of vitamin A in alcohol.¹⁷⁷ It has been recommended¹⁸⁰ to check the instruments used against a suitable potassium chromate solution as a standard before and after each determination in order to increase the accuracy of the determinations. The factor for converting $E_{1\text{cm}}^{1\%}$ 328 m μ into International Units of vitamin A per gram is 1600, as accepted by the International Vitamin Conference 1934 (conversion factor' = I U / $E_{1\text{cm}}^{1\%}$)¹⁷⁸. The value for the conversion factor for pure vitamin A is believed to be about 2000-2100¹⁷⁹ or even 2375 but not enough experimental data are available to decide which value is correct.^{18 183} For vitamin A preparations from whale livers a conversion factor $E_{1\text{cm}}^{1\%}$ 325 m μ of 1200 has been recommended¹⁸⁴ due to the presence of considerable amounts of materials such as vitamin A₃.

A certain amount of the observed total absorption at 325-328 m μ may be due to carotenoids other than vitamin A and especially to glycerides. This is of importance when determinations of low potency materials are made whereas the error becomes negligible with preparations of high vitamin A potency.

As stated before vitamin A and the provitamins are destroyed by ultra violet light. Therefore, the determination of the absorption band is somewhat inexact. At very low temperatures a differentiation between the band of vitamin A and the irradiation product of carotene is possible.¹⁸¹ The selective decrease of absorption at 325 m μ produced by irradiating an alcoholic solution of a marine fish liver oil with mercury light of 365 m μ which destroys vitamin A, is said to be an accurate measure of the vita-

¹⁷⁷ F. L. Smith, B. E. Stern and F. L. Young, *Nature* 141, 55 (1938). E. M. Hume and H. Chicks, *Med. Research Council Brit. Special Rept. Series* 1935, No. 202.

¹⁷⁸ See also E. M. Hume, *Nature* 139, 467 (1937).

¹⁷⁹ H. N. Holmes and R. E. Corbet, *J. Am. Chem. Soc.* 59, 2043 (1937). T. H. Mead, S. W. I. Underhill and K. H. Coward, *Biochem. J.* 33, 589 (1939).

¹⁸⁰ J. B. Wilke, *J. Assoc. Official Ag. Chem.* 22, 465 (1939).

¹⁸¹ F. P. Bowden, S. D. D. Morris and C. I. Snow, *Nature* 131, 58 (1933).

¹⁸² This high factor could, for example, be caused by a hyper activity of the U. S. Pharmacopoeia Reference Oil or by an inadequate utilization of carotene (less than 50%) when the U. S. Pharmacopoeia Reference Oil was initially standardized. It has also been debated if the values are not exaggerated due to deterioration of the Standard Reference Oil. *J. R. Edisbury, Analyst* 65, 484 (1940).

¹⁸³ J. G. Baxter and C. D. Robeson, *Science* 92, 707 (1940).

¹⁸⁴ J. R. Edisbury, *Analyst* 65, 484 (1940).

min¹⁸⁵ The effect on other constituents, particularly, carotenoids is however, unpredictable

Fluorescence offers a possible method of differentiating between vitamins A₁ and A₂ Vitamin A₁ exhibits a characteristic green fluorescence, vitamin A₂ a reddish fluorescence¹⁸⁶ Upon irradiation both fluorescences fade

(c) *Biological Methods*

The efficacy of vitamin A preparations is tested on young rats In the prophylactic method the substance with the unknown vitamin A content is added to a vitamin A free diet, and the growth of rats is compared with the growth rate of normally growing animals Another method uses young rats which have ceased growing due to a vitamin A deficiency but gain weight after the addition of vitamin A The results are compared with an International Standard preparation of β carotene, which, as has been pointed out before is converted under suitable conditions in the rat into an equimolecular quantity of vitamin A The latter method is recommended by the U S Pharmacopoeia

In addition the appearance of a cornified epithelium in the vaginas of spayed rats (colpokeratosis) on a vitamin free diet and its rapid disappearance following vitamin A feeding have been used for the determination of vitamin A

Another biological test which has been used for determining the physiological minimum requirement is the evaluation of the dark adaptation of the eye after exposure to bright light Individuals who have a partial or complete deficiency of this vitamin show some degree of night blindness¹⁸⁷

21 Standards

The International Standard for vitamin A was adopted by the International Vitamin Conference 1934 and subsequently adopted for the United States Pharmacopoeia

¹⁸⁵ A Chevallier *Z Vitaminforsch* 7 10 (1938) N K De *Indian J Med Res* 24 3 (1937)
O Notewark *Biochem J* 29 12 7 (1935)

¹⁸⁶ R Greenberg and H Popper *Proc Am Physiol Soc* 1940 71

¹⁸⁷ See chapter on the visual purple This method was originally described by P C Jean and J Gentile *J Am Med Assoc* 102 89 (1934) and has been modified by several workers

1 I U (International Unit) = I U S P Unit

= 0.6 γ ¹²⁴ of pure β carotene m p 184 optically inactive dissolved in coconut oil¹²⁵ with addition of hydroquinone

= 10-2 Sherman Units

1 g of U S P cod liver oil must contain at least 850 U S P Units of vitamin A

1 g pure carotene contains 1 670 000 I U

1 g pure vitamin A contains 4 500 000 I U

1 C L O Unit (Cod Liver Oil Unit) = 12.7 γ β -carotene

= 208 U S P Units

= 10 Lovibond Units

= 50 Lovibond Units (Wolff)

= 550 blue units (Moore)

1 blue unit (Moore) = Carr Price value 0.0182

= $E_{1\text{cm}}^{1\%}$ 328 m μ 0.000373

= 60 I U

The ratio of $E_{1\text{cm}}^{1\%}$ 620 m μ to $E_{1\text{cm}}^{1\%}$ 620 m μ (L —620 m μ value) is 1.30 ± 0.03

The ratio of the activity of β carotene and of vitamin A as reported above has been determined under the specified conditions of the biological test. An equality of an International Unit of vitamin A and of carotene can be claimed only under those conditions and is actually quite different for the metabolism of a normal growing organism. To express the requirement of an organism, double standards must be recognized—one for carotene and another one for vitamin A (see also page 95). (For a discussion of the various influences on vitamin A and on carotene utilization by the organism see page 84.)

At the level of a physiological optimum the ratio of efficiency of vitamin A to carotene by weight is about 6:1 and at a level that assures significant storage and successful reproduction about 10:1.

It would be well to express the values of vitamin A potency not only in units but it should be required to indicate the method which was used for the determination—for example U S Pharmacopoeia method, spectroscopic method, etc.

22 Physiology of Plants and Microorganisms¹²⁶

Plants and also microorganisms contain only provitamins A. The presence of vitamin A has never been demonstrated and there is ample

¹²⁴ 1 γ = 0.001 mg = 10 μ g

¹²⁵ The standard of 0.6 γ β carotene as an International Unit replaced the earlier standard set up in 1931. The standard Unit was then defined by agreement to represent 1 γ of carotene m p 179 prepared according to the method of Willstätter. Willstätter became evident that carotene is a mixture of varying amounts of some compound; the biological equivalent of the earlier standard expressed in weight of the pure β -carotene was adopted as International Standard.

¹²⁶ See also O. A. Bessey and S. B. Wolbach *J Am Med Assoc* 110:207 (1937).

evidence that plants and many microorganisms do not need this vitamin. Whatever the function of this vitamin may be in the animal organism, either plants do not need this function or they take care of it by some other means. On the other hand, the relatively high concentrations of carotenoids and especially of provitamins in plants are striking. These compounds generally appear in all growing parts of the plant and especially in the shoots. It thus appears that provitamins A have a very definite purpose in the plant organism. What this is, has however, not been elucidated. It is suspected that plants need provitamins A for proper growth. It has also been assumed that at least part of the action of these compounds is related to the efficient utilization of light by the plant organism. Plants are definitely photosensitive and phototropic responses have been shown to be associated with the presence of carotenoids.¹⁹¹

23 Animal Physiology

(a) Metabolism of Provitamins A and of Vitamins A

Both provitamins A and vitamins A are preferably administered orally although they can be applied parenterally, for example, by subcutaneous or intramuscular injection. They are also efficiently absorbed through the skin.¹⁹²

Provitamins A and vitamins A are absorbed from the intestinal tract. Carotenes in colloidal water solutions are efficiently utilized, for example, by the rat¹⁹³ to prevent vitamin A deficiency symptoms. Proper absorption of vitamin A is on the other hand related to the presence of fat. The nature of the fat or oil, which is used for example, for dissolving the vitamin, determines to a certain extent the degree of utilization of the vitamin. The more unsaturated the oil is, the better the absorption. From mineral oils, vitamin A is absorbed only sparingly. The absorption of provitamins A and of vitamins A is furthermore linked to the presence of bile acids and apparently also of pancreatic lipase. In jaundice and in choledochocolonostomized dogs carotene is not absorbed.¹⁹⁴ There is evidence that the absorption mechanism of birds is somewhat different in that these animals need some external supply of fat for proper utilization of

¹⁹¹ E. S. Johnston *Smithsonian Inst. Pub.* 92: 11 (1934). G. Wald and H. G. du Buy *Science* 84: 47 (1936).

¹⁹² W. v. Drigalski *Z. Vitaminforsch.* 3: 200 (1934). H. J. I. uber und H. Rocholl *Klin. Wochschr.* 11: 1143-1709 (1935).

¹⁹³ B. N. Majumdar *Ind. J. Med. Res.* 27: 413 (1939).

¹⁹⁴ J. D. Creaves and C. L. A. Schmidt *Am. J. Physiol.* 111: 499-502 (1935).

carotenes while vitamin A is apparently absorbed efficiently. practically no fat is given in the diet ¹⁸

The absorption of provitamins and of vitamins A from the tract is a rapid process. The absorption of vitamin A in rats reaches a maximum in three to five hours after administration, at the disappearance from the intestinal tract and the rise of the concentration in the blood ¹⁹⁶. The rate of absorption of the provitamins A is somewhat slower and reaches its maximum at seven to eight hours after administration. Vitamin A esters are hydrolyzed in the gut prior to absorption, ¹⁹⁷ but it seems that the vitamin is re-esterified during or soon after the absorption ¹⁹⁸.

Both provitamins A and vitamins A are transported through the organism by the blood. The carotenoids are present in the blood serum while the erythrocytes are devoid of these compounds. They appear to be solubilized by the formation of loose protein addition compounds. There seems to exist a minimum normal blood level of vitamin A and perhaps also of provitamin A. This level is independent of the amount of stored vitamin or provitamin and is only temporarily increased during times of absorption from the intestines. In vitamin A depleted rats for example, the vitamin A content of the blood plasma is zero. During times of low vitamin A intake, the level in the blood is directly related to the amount fed. After the concentration in the serum has reached what appears to be an optimal level that is about 100 International Units per 100 cc of plasma the concentration does not increase in the blood even when excessive amounts are given. On the other hand no vitamin A is stored during low vitamin A intake while this vitamin is rapidly deposited in the liver ¹⁹⁹ when optimal doses are administered.

Provitamins A and vitamins A are removed from the blood by the reticulo endothelial system ²⁰⁰. Large amounts of vitamin A are stored in the liver. There are no quantitative data available which would indicate what the minimum amount stored in the livers of any species or of man should be. The vitamin is present in the liver mainly in the Kupffer and epithelial cells in the latter in lipid droplets and diffusely in the cytoplasm. Vitamin A is also stored to a certain extent in the adrenal cortex.

¹⁸ W C Russell M W Taylor H A Walker and I J Pol kn P oc Am Soc B l Chem 1941 CIX

¹⁹⁶ S W Cl usen J Am Med Assoc 101 1384 (1933)

¹⁹⁷ B L Gray K Morgareidge and J D Cawley P oc Am Soc Biol Chem 1940 XXXVII

¹⁹⁸ J C. Drummond M F Bell and T T Palmer B st Med J I 1908 (193)

¹⁹⁹ J M Lew s O Bodansky K F Falk nd G McGu te, Proc Soc Exptl B cl Med 46 48 (1941)

²⁰⁰ B Ahmad Ck s t Sci 2 477 (1934) F Larch and D Roller Klin Wochsch 15 1636 (1936)

in the form of small globules, in the *corpus luteum* and in the lutein cells of the ovary. Generally, fat cells contain small amounts. Traces may be found in the interstitium of the renal cortex and papilla, in the alveolar septum of the lungs and the intermediary part of the pituitary.²⁰¹

Vitamin A is stored in the body mainly in esterified form, although small but definite amounts of the free vitamin are always present. The stored vitamin A esters are derivatives of a series of saturated and unsaturated fatty acids. Whether or not certain specific acids are preferentially selected by the organism for esterification purposes is not known.²⁰²

The ability of the animal organism to store large amounts of vitamin A has been discussed. The rat, for example, may store in a few days enough vitamin A to supply its nutritional needs for several months.²⁰³ In the mammal this storage is very efficient after times of vitamin A depletion or of low vitamin A intake. The efficiency of this storage decreases, however, with an excess intake over the minimum physiological requirement.²⁰⁴ On the other hand, the stored vitamin A is used rapidly when the food intake is lacking in this vitamin until a certain apparently critical vitamin A level in the liver is reached. Thereafter, the rate of depletion is much slower.²⁰⁵ These observations suggest that a special vitamin A utilization mechanism exists in the organism. Provitamins A are stored in small but definite amounts. Examples have been given in the section on the occurrence of provitamins A (see page 38).

Vitamin A and to a certain but considerably smaller extent also provitamins A are secreted into milk. In cow's milk, the ratio of vitamin A to provitamin A varies considerably with the species, breed, nutrition, etc., but the total is of the same order of magnitude regardless of the breed.²⁰⁶ The vitamin A activity of the milk is fairly constant regardless of the time of the day when the milk is secreted, but is to a certain extent influenced by the vitamin A potency of the food. Human milk is about 5 to 10 times as rich in vitamin A potency as cow's milk. The colostrum of all species investigated contains a much higher vitamin A potency than the milk. In the case of humans the colostrum contains 2 to 3 times, and in the case of cows 10 to 100 times the amount present in milk.²⁰⁷ Both provitamins and vitamins A are also secreted in eggs. A special mechanism apparently

²⁰¹ H. Popper and R. Greenberg *Proc Am Physiol Soc* 1940 14f

²⁰² E. L. Cray, K. C. D. Hickman and F. F. Brown *J Nut Ion* 19 39 (1940)

²⁰³ A. W. Davies and T. Moore *Biochem J* 31 172 (1937)

²⁰⁴ L. F. Booher and M. B. Porter *Proc Am Soc Biol Chem* 1941 XCI

²⁰⁵ A. W. Davies and T. Moore *Biochem J* 29 147 (1935)

²⁰⁶ C. A. Baumann, H. Steenbock, W. M. Deen and I. W. Rupel *J Biol Chem* 105 1167 (1934)

²⁰⁷ W. J. Dann *Biochem J* 30 1644 (1936)

exists for the passage of vitamin A through the placental wall. The vitamin A content of all newborn animals is low but a vitamin A deficiency has never been found when the mothers have had an ample supply of this vitamin. After birth the vitamin A content rises rapidly and reaches the normal level of the adult within a few days or weeks.

Both provitamins and vitamins A are in general readily metabolized. No excretion takes place through the kidneys. In the feces a certain proportion of the ingested provitamins may be found, especially when excessive amounts are fed or when the absorption mechanism is hampered.

(b) *Physiological Action of Provitamins A and of Vitamins A*

The physiology of man and of animals as influenced by vitamin A must ultimately be traced to the mechanism of the vitamin A action. This mechanism is for the most part unknown.

It has already been discussed that provitamins A are absorbed in the intestinal tract and to a certain extent stored in the animal organism. It has also been shown that provitamins A are converted in the organism into vitamin A. Except as precursor of vitamin A, carotenes as such are not known to be active in the animal economy. The possibility should, however, not be overlooked that provitamins may act in a specific way of their own. Thus it may be significant that provitamins A are stored in man and in animals in a number of special organs, primarily in the glands which are concerned with the functions of reproduction. In invertebrata which are able to synthesize their own specific carotenoids^{208, 209} these carotenoids apparently play a definite and important role in metabolic processes. It has also been suggested that, for example, in mussels, carotenoids may play some role in gametogenesis.⁹⁹ In this connection it is well to recall the action of various carotenoids of algae for which these compounds are secretion products concerned with the development of motility and with conjugation of the gametes.¹⁰ While these carotenoids in lower animals are not necessarily provitamins A, they are closely related to them. Thus, it seems possible that the provitamins A have an influence upon the maintenance of a normal mechanism of the sex apparatus. It has furthermore been shown repeatedly that the sex organs of rats kept on a vitamin A depleted diet degenerate. This may of course be explained as meaning that either vitamin A or provitamins A are necessary. It is however also possible that the sex glands do not utilize the provitamins A proper but

²⁰⁸ E. Ledere, *Bull. soc. chim. bel.* 20: 554-567, 611 (1938).

²⁰⁹ B. T. Scher, *J. Biol. Chem.* 136: 75 (1940).

¹⁰ F. Moewus, *Naturwissenschaften* 27: 97 (1933).

have a mechanism of their own for converting the provitamins into active compounds such as, for example vitamin A

Another unsettled question of physiological importance is the problem of whether or not the vitamin A itself acts in the organism. It has previously been stated that vitamin A is stored in the liver mainly in the esterified form, but that some free vitamin A can always be found. Thus it is possible that the vitamin is esterified only for the purpose of storage and it is probable that the vitamin acts in the free form. This question cannot be decided definitely until the mechanism of the vitamin A action has been elucidated.

A possible clue to this mechanism may be seen in the increase of purines in the growing vitamin A depleted tissue after this vitamin has been administered.²¹ Purines are necessary building units of cell nuclei. Actually all primary and secondary symptoms of a vitamin A deficiency can be explained on this basis. They all constitute cellular changes in the most sensitive parts of the body, such as in the respiratory mucosa, the salivary glands, the intestinal tract and finally in the skin in general. Thus, the principal role of vitamin A is to stimulate the building of cells.

Beyond this fundamental and specific action of vitamin A a general influence on the basic metabolism has also been considered. A possible connection with the oxidation mechanism has been suggested and was apparently supported by experiments which showed that the oxygen consumption of livers in the presence of iron containing porphyrines increases with the amount of vitamin A present.²² This theory has not however, been further advanced and appears to be somewhat doubtful and unspecific in view of the fact that it has been possible to connect some of the other vitamins with definite stages of the oxidation reduction mechanism of the living tissue. This has however, not been possible in the case of vitamin A.

Vitamin A seems to exert however, some non specific functions upon the fat and carbohydrate metabolism. The necessity of the presence of fat has been discussed in connection with the absorption and storage of this vitamin. Furthermore during avitaminosis the amount of fat deposited in the organism decreases slowly but the fat deposits are restored upon administration of vitamin A. Similarly the cholesterol content in the organism decreases during times of low vitamin A intake. On the other hand, excess doses of vitamin A cause an appreciable increase of the cholesterol content in blood and in the brain. Similar relations have been

²¹ H. v. Euler and G. Schmidt *Z. physiol. Chem.* 23: 215 (1934)

²² H. v. Euler and L. Ahlström *Ibid.* 204: 168 (1932)

found for the carbohydrate metabolism for example for the glycogen content of the liver

Vitamin A acts, however, in the body also in some other specific way. In conjunction with a specific protein vitamin A plays an essential role in the visual purple. This will be discussed in a special section (see below). Whether or not vitamin A acts in conjunction with other proteins in some other specific functions is not known. It has been found experimentally that in vitamin A deficiency a marked decrease in the concentration of blood serum esterase, an appreciable decrease in hepatic esterase and a marked increase in hepatic lipase occur.¹³ These findings need confirmation and should not necessarily be interpreted as indicating a relationship of vitamin A to specific enzyme systems.

Vitamin A is mobilized in the organism in a very special way whenever a state of disease occurs. Thus the intake of ethyl alcohol by dogs²¹⁴ or by human beings causes a specific mobilization of the vitamin A stored in tissues since both the blood level²¹⁵ and the dark adaptation test²¹⁶ indicate higher vitamin A concentrations than normal. In many diseases such as fever etc. an increased demand for vitamin A has been demonstrated.²¹⁷ The turnover of vitamin A in the liver can also be effected by injection of carcinogenic compounds, such as dibenzanthrene, benzopyrene and methylcholanthrene.²¹⁸ This effect is not necessarily specific for vitamin A although it appears to be.

(c) *The Visual Purple*

The energy of dim light, which strikes the eye, is transformed into nerve impulses by the visual purple on the outside end of the retina. Color and light of high intensity are perceived by the cones of the retina.

Rhodopsin, the visual purple, bleaches out under the influence of light and regenerates in the absence of light. Rhodopsin is a carotenoid albumin, with an absorption maximum at 500 $\mu\mu$. The prosthetic group, retinene, is a carotenoid of unknown composition,¹⁹ but related to a form

¹³ B. Sure, M. C. K. K. and K. S. Bucian, *Proc. Soc. Exptl. Biol. Med.* **35**, 209 (1936).

¹⁴ S. W. Clausen, W. B. Baum, A. B. McCoord, J. O. Ryden and B. B. Beebe, *Science* **91**, 318 (1940).

¹⁵ S. W. Clausen, B. B. Beebe, W. S. Baum, A. B. McCoord and J. O. Ryden, *Ibid.* **93**, 21 (1941).

¹⁶ L. B. Pett, *Ibid.* **92**, 63 (1940).

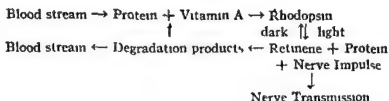
S. W. Clausen, *J. Am. Med. Assoc.* **111**, 144 (1935).

¹⁷ C. A. B. Umann and F. G. Foster, *Proc. Am. Soc. Biol. Chem.* **1941**, 111.

¹⁸ L. S. Frerking and E. Holm, *Am. J. Physiol.* **73**, 63 (1925).
¹⁹ S. Hecht, A. M. Chase, S. Shlaer and C. Haig, *Science* **84**, 331 (1936).
²⁰ S. Hecht, *Physiol. Rev.* **17**, 239 (1937).
²¹ S. Hecht, A. M. Chase and S. Shlaer, *Science* **85**, 567 (1937).
²² C. Haig, S. Hecht and A. J. Patek, *Ibid.* **86**, 534 (1937).
²³ S. Hecht and J. Mandelbaum, *Ibid.* **88**, 219 (1938).
²⁴ S. Hecht and J.

(Footnote continued on page 90)

of carotene Under the influence of light the red color disappears and an unstable orange compound transient orange, is formed This changes probably in a dark process, to indicator yellow which appears to be a loose protein compound of retinene Retinene can be extracted from the visual yellow with chloroform, is ligroin soluble and has an absorption maximum at 385 $m\mu$ in chloroform ($SbCl_5$ color reaction yields a maximum at 664 $m\mu$) Retinene can also be obtained from rhodopsin through acid or alkali hydrolysis The visual yellow breaks down further to vitamin A and a specific protein In the dark, a partial resynthesis of rhodopsin occurs The pigmented layers of the eye contain large amounts of vitamin A, which is used for the synthesis of rhodopsin Vitamin A deficiency therefore causes as one of its first symptoms a decreased ability for dark adaptation since not enough vitamin A is available for the synthesis of rhodopsin



Vitamin A is replaced in fresh water fish by vitamin A which replaces also the vitamin A in the visual purple and is called porphyropsin (absorption maximum at 525 $m\mu$)²²⁰ In some marine fish compounds of different absorption characteristics are found which indicate the presence of additional factors A (or mixtures of rhodopsin and porphyropsin)²²¹

(d) Relation of Vitamins A to Other Vitamins, Hormones, etc

In the general chapter on the interrelationship between the vitamins and the hormones it has been pointed out that the physiological equilibrium of any organism is disturbed if one of the vitamins is not supplied regularly and adequately

Of special interest in the case of vitamin A are the claims concerning an antagonism with the other fat soluble vitamins especially vitamin D and the antagonism with some of the water soluble vitamins especially vitamin C It has been stated that a vitamin A deficiency causes similar

²²⁰ (Continued)

Mandelbaum *Am J Physiol* 130 451 (1940) C Wald *J Gen Physiol* 18 905 (1935) G Wald and A B Clark *Am J Physiol* 116 157 (1936) G Wald *J Gen Physiol* 19 351 781 (1935) 20 45 (1936) *Nature* 139 587 (1937) R J Lythgoe *J Physiol* 89 331 (1937)

²²¹ C Wald *Nature* 139 1017 (1937)

²²² C Wald *ibid* 139 1017 (1937) R J Lythgoe and K. Tanley *Proc Roy Soc (London)* B120 95 (1936)

disturbances as an excess supply of vitamin D. The vitamins A and D however do not exert any synergistic or antagonistic action at the normal optimal concentrations. The natural combination of vitamins A and D in liver oils prevents any signs of hypervitaminosis even if given in large amounts due to the alleged antagonism of these vitamins which in this case even represents a synergism. Antagonism of vitamins A and C is seen when both vitamins are given in excess at the same time since no vitamin A hypervitaminosis develops.^{2, 3}

There is apparently a relationship between the vitamin A metabolism and vitamin E. During vitamin E deficiency in the rat no vitamin A is stored in the liver and the amount of vitamin reserves in the liver is markedly reduced even when ample quantities of vitamin A are administered.^{22, 24} Thus an acute secondary deficiency of vitamin A develops. Whether this is due only to the non specific antioxidant power of vitamin E compounds or to a specific physiological effect is not known.

Among the relations of vitamin A to hormones the alleged antagonism to the hormone of the thyroid gland thyroxine has been extensively studied.²⁴ Toxic effects of excesses of this hormone are said to be overcome by the administration of large amounts of vitamin A. Rats given thyroxine were depleted of vitamin A much more rapidly than normal rats.²² No evidence for a specific antagonism could be obtained, however,²⁶ but a temporary disturbance in the metabolic rate has been found upon administration of large doses of vitamin A to normal and thyroidectomized rats.²

The secretion of the hormones of the anterior lobe of the pituitary gland seems to be related to the vitamin A metabolism since in frogs the glycotropic hormone could be detected in the liver only after intake of vitamin A.^{2, 8} together with vitamin E.

24 Avitaminosis and Hypovitaminosis

The action of vitamin A can be regarded essentially as a stimulus to the building of new cells. Thus vitamin A deficiency causes retarded growth. This symptom is however not specific for a vitamin A deficiency.

Specifically, lack of vitamin A causes atrophy of the epithelium with substitution of a stratified keratinized epithelium for the normal epithelial

²² A Scheunert *Naturwissenschaften* 28 297 (1940)

²³ H W Nodt and H Schroeder *Z Vitaminforsch* 4 206 (1933)

²⁴ T Moore *Biochem J* 34 1391 (1940) A W Davies and T Moore *Vitam* 147 794 (1941)

H v Fuler and E Klusmann *Z physiol Chem* 213 21 (1932)

²⁵ J D Greaves and C I A Schmidt *Am J Physiol* 116 456 (1936)

²⁶ C H Baum and T Moore *Biochem J* 33 1639 (1939)

²⁷ R F Sheets and H C Struck *Proc Am Phy Soc* 1941 26

²⁸ L Kerpov *Compt rend* 209 358 (1939) 210 188 (1940)

structure²²⁹ This is first observed in the respiratory mucosa The mucosa in the mouth and the salivary glands is next affected causing in turn a greater susceptibility to infections Finally the epithelial mucosa of the eyes, the intestinal tract the urethra, the kidney etc is degenerated The epithelium of the vagina is especially sensitive to vitamin A deficiency causing the appearance of colpokeratosis and of the so called senile vaginitis²³⁰ In man a general dryness of the skin and a hyperkeratosis of the hair follicles are observed The hair becomes dry and lusterless Dermatitis a form of keratosis or cutaneous eruption, occurs on the fore arms and thighs and later practically all over the body²³¹ Gastrointestinal disorders may occur³ and stones may be formed in the bladder and in the kidney (urinary calculi)²³² In some experimental animals for example, in pigs and in rats certain neurological lesions also were observed²³⁴

The various eye lesions caused by vitamin A deficiency are especially significant An early symptom of a hypovitaminosis is often the so called night blindness (nyctalopia or functional hemeralopia)²³⁵ At later stages of the deficiency a softening of the cornea, followed by perforation (keratomalacia) and a dry, lusterless condition with white deposits on the scleral conjunctiva (nutritional xerophthalmia) occur Vitamin A deficiency may also contribute to the development of myopia²³⁶ In pigs from sows kept on a vitamin A depleted diet during pregnancy total blindness was observed and some animals had no eyes at all²³⁷ Lack of vitamin A may cause blindness by a constriction of the optic nerve associated with a stenosis of the optic canal²³⁸

During times of vitamin A deficiency, the normal functioning of the reproductive system is hampered In the female rat, the vaginal mucous membrane becomes cornified, as previously stated There is also some adverse influence on the ovary, and normal reproduction does not occur

²²⁹ S B Wolbach and P R Howe *J Exptl Med* 42 763 (1927)

²³⁰ J W Simpson and E E Mason *Am J Obstet Gynecol* 32 127 (1936)

²³¹ L J A Löwenthal *Arch Dermat Syphilol* 28 700 (1933) C N Frazer and C K Hu *Ibid* 33 825 (1936) J B Youmans and N B Corlette *Am J Med Sci* 195 644 (1938)

²³² R Roller *Z Klin Med* 130 163 (1936) G Will *Klin Wochschr* 15 1281 (1936)

²³³ W M Kerns *Wisconsin Med J* 36 170 (1937) C C Higgins *J Urol* 36 168 (1937) *Sw g Gynecol Obstet* 63 23 (1936) M Meltzer *N Y State J Med* 37 865 (1937)

²³⁴ S B Wolbach and O A Bessey *Science* 91 599 (1940)

²³⁵ I O Park *J Oklahoma Med Assoc* 28 357 (1935) 29 123 (1936) P C Jeans and Z Zentmire *J Am Med Assoc* 106 996 (1936) P C Jeans E Blanchard and Z Zentmire *Ibid* 108 451 (1937)

²³⁶ H Frandsen *Acta Ophthalmol (Supplements)* 4 (1945) H Jeghers *New Engl J Med* 216 51 (1937) *Ann Internal Med* 10 1304 (1937)

²³⁷ H Miller *Am J Ophthalmol* 23 296 (1940)

²³⁸ J L Novaes *Hora Medica* 11 84 (1939)

²³⁹ L A Moore *J Nutrition* 17 443 (1939)

In the male rat, the testes degenerate in severe cases of A avitaminosis

Vitamin A deficiency during the period of tooth development can impair tooth structure by causing an atrophy and metaplasia of the enamel organ.²³⁹ Thus a hypoplastic tooth with thin defective enamel is formed. The rate of apposition of dentine is altered in vitamin A deficiency while the life span of the formative cells is not affected.⁴⁰ In rats the incision teeth lose the deep orange pigment in the enamel which is restored upon administration of vitamin A.²⁴¹

An early symptom of vitamin A deficiency is the decrease of the normal vitamin A and carotenoid level in blood. The normal level for children, when determined under specified conditions is from 5.5 to 27.3 units of vitamin A per cc of blood and the carotene level is from 3.1 to 75 units per cc. A vitamin A level of less than 3 units of vitamin A indicates avitaminosis and a value of less than 3 units of carotenes points to a state of hypovitaminosis.²⁴² During avitaminosis vascular lesions in practically the entire arterial system have also been observed.²⁴³

The state of hypovitaminosis or avitaminosis may be caused besides by an insufficient intake of vitamin A also by an impaired intestinal absorption as has been found in cases of congenital obliteration of the bile ducts, fibrosis of the pancreas, celiac disease and others.²⁴

(a) Clinical Test Methods

1. **The Dark Adaptation Test** Night blindness in the absence of eye disease and of a hereditary tendency in that direction may often be a manifestation of vitamin A deficiency.²⁴⁴ For actual diagnosis a number of different adaptometers²⁴ which are modified photometers are available on the market. The test is carried out by one of two methods. The patient after a preliminary period in the dark is exposed to subdued light and the minimum amount of light that is visible is measured. This method has been found especially useful for the determination of a vitamin A deficiency in infants.²⁴⁵ In the other method the visual purple in the

²³⁹ S. B. Wolbach and P. P. Howe *J. Exptl. Med.* 42: 753 (1935).

²⁴⁰ L. Schour, M. C. Smith and M. M. Hoffman *Proc. Soc. Exptl. Biol. Med.* 39: 447 (1938).

²⁴¹ J. T. Irving and M. B. Richards *Nature* 144: 908 (1939).

²⁴² C. D. May, K. D. Blackfan, J. F. McCreary and F. H. Allen *Am. J. Diseases Children* 59: 1167 (1940).

²⁴³ L. Oppel *Proc. Soc. Exptl. Biol. Med.* 40: 449 (1939).

²⁴⁴ J. B. Feldman *Arch. Ophthalmol.* 12: 81 (1934); 15: 1004 (1936); 17: 648 (1937); 18: 821 (1937).

²⁴⁵ G. S. Derby, P. A. Chandler and L. L. Sloan *Arch. Ophthalmol.* 3: 31 (1930). S. Hecht and J. Mandelbaum *J. Am. Med. Assoc.* 112: 1910 (1930). S. Hecht and S. Shlaer *J. Optical Soc. Am.* 28: 269 (1938).

²⁴⁶ C. Fredericksen and C. Edmund *Am. J. Diseases Children* 53: 89: 1179 (1937).

retina is bleached by means of bright light, and the minimum time necessary to recover clear vision is then determined ²⁴⁷

2 Determination of the Vitamin A Content of Blood A number of different methods have been developed for the determination of the vitamin A content of blood. Assays can be made with the serum alone since the blood corpuscles do not contain significant amounts of this vitamin. The actual determination can be carried out spectroscopically in alcohol solution after the blood has been treated with sodium sulfate ²⁴⁸. It has also been recommended to test for vitamin A in blood by determining the amount of material destroyed by ultraviolet light ⁴⁹. For this purpose the blood is hydrolyzed and the non saponifiable fraction is determined spectroscopically for its apparent vitamin A content. Irradiation with monochromatic light of 365 m μ causes specific changes of the absorption maximum of vitamin A from which the actual amount of vitamin A present in the solution can be calculated. This method cannot be used, however, if appreciable amounts of carotene are present. The best method for determining both vitamin A and provitamins A in blood consists in a combination of a spectroscopical determination with the Carr Price color reaction ²⁵⁰. For this purpose, blood serum is extracted with a mixture of ethanol and petroleum ether ⁵¹. The petroleum ether extract is used for a spectroscopical determination of the carotenoids²⁵² since bile pigments are not extracted under the specified conditions. For practical purposes the total carotenoid value is considered to consist of a 50-50 mixture of β carotene with the inactive xanthophyll. The vitamin A content of the extract is then determined according to the Carr Price technic (see page 78). The vitamin A content of blood can of course also be determined by the biological methods described for the determination of vitamin A.

3 Demonstration of Keratinized Epithelial Cells In this test the presence of cornified cells in scrapings from the cornea, nose and mouth and in secretions from the trachea, bronchi, kidneys and vagina is investigated by special staining methods which give the keratinized cells definite colors while leaving normal cells colorless. Several reagents have been suggested, among which is 1% methylene blue in a 3% acetic

²⁴⁷ L. B. Pett, *J. Lab. Clin. Med.* 25: 141 (1931).

²⁴⁸ A. Chevallier and Y. Choron, *C. r. Acad. Sci. Paris* 118: 883 (1917).

⁴⁹ A. Chevallier, Y. Choron and R. Matheron, *Ibid.* 127: 241 (1918).

²⁵⁰ C. D. May, K. D. Blackfan, J. F. McCreary and F. H. Allen, *Am. J. Diseases Children* 59: 111 (1940).

⁵¹ W. Clausen and I. A. B. McCoord, *J. Biol. Chem.* 13: 673 (1918).

²⁵² W. S. Ferguson, *Analyt.* 60: 680 (1917).

acid water solution (Mallory's stain for diphtheria bacilli) which dyes the pathological cells deep red.⁵²

4 Vitamin A Absorption Test The efficiency of the intestinal absorption of vitamin A is studied in this test.⁵⁴ A standardized test dose of vitamin A is given by mouth and the vitamin level of the patient's blood is assayed before and at definite intervals after the vitamin intake. An average rise of at least 50 to 130 units of vitamin A per cc should be observed in from three to five hours; otherwise a subnormal efficiency of the intestinal absorption is indicated.

25 Hypervitaminosis

No indications are known for any toxic effects of excessive doses of provitamins A. It can be assumed that a regulatory mechanism in the body takes care of handling amounts of provitamin A which are in excess of the animal's actual needs.

The toxicity of pure crystalline vitamin A has not been investigated so far. Using vitamin A concentrates, doses in excess of 100,000 units per day are harmful for rats.⁵⁵⁻⁵⁶ The pathological changes observed include retarded growth, hemorrhages, especially in the mucous membranes, and abnormal rarefaction and fragility of the bones.

26 Vitamin A Requirements

All mammals, birds, and fish which have been investigated, utilize vitamin A. This does not mean, however, that all animals need this factor. Thus, it has been demonstrated⁵⁷ that the cockroach (*Blattella germanica* L.) needs no provitamin or vitamin A in the diet and is able to function normally throughout life without this vitamin. It is therefore suspected that other animals, such as certain insects, do not require vitamin A.

Double requirement standards must be recognized, one for vitamin A and one for carotene (provitamin A) since the International Units of carotene and of vitamin A are equal only under the specified conditions of the biological test, but not in the metabolism of a normal growing organism.⁵⁸

The vitamin A requirements of adult mammals can be correlated to the body weight. Fairly uniform requirements have been established for

⁵² K. D. Blackfan and S. B. Wolbach, *J. Pediat.* **3**: 66 (1933).

⁵³ J. Chesney and A. B. McCord, *J. or Soc. Exptl. Biol. Med.* **31**: 88 (1934).

⁵⁴ F. B. Vedder and C. Rose, *berg J. Nutrit.* **16**: 5 (1938).

⁵⁵ I. Ikegaki, *Z. Vitaminforsch.* **7**: 113 (1938).

⁵⁶ R. I. Bowra and C. M. M. Cay, *Science* **92**: 271 (1940).

⁵⁷ H. R. Gilbert, C. F. Howell, and G. H. Hart, *J. Nutrit.* **19**: 91 (1940).

man²⁵⁹ horse,²⁵⁸ dog,²⁶⁰ cattle,^{258, 61} sheep,^{258, 261} swine,^{258, 261, 262} rabbit,²⁶³ rat⁶⁴ and hedgehog²⁶⁵ All show approximately the same requirements, namely, 25 γ of β carotene (corresponding to 40 International Units) or 4 γ (20 International Units) of vitamin A per kilogram of body weight^{258, 261, 266} These values represent the minimum for normal growth without showing any clinical symptoms of vitamin A deficiency, but little or no storage of the vitamin occurs at these levels About three times the minimum amount of vitamin A (12 γ or 60 International Units) and five times the minimum amount of β -carotene (125 γ or 200 International Units) is considered minimum for significant storage and reproduction The optimum vitamin A requirement for an average adult is then about 5000 International Units of vitamin A or 15 000 International Units of carotene Increased amounts are recommended for pregnant and nursing women and during adolescence⁶⁷ The allowances as recommended by the Food and Nutrition Board of the National Research Council are reprinted on page 613

The vitamin A requirements of animals other than mammals are less well known Birds apparently need vitamin A in an amount of the same order of magnitude as mammals Growing chicks need about 95 to 125 γ of carotene per day⁶⁸ (or about 1800 International Units of vitamin A per pound of feed) but for laying stock 200–500 γ of carotene have been recommended²⁶⁹ (This amount corresponds to a diet of yellow corn or to an addition of 2.5–5% of a good grade alfalfa meal)

²⁵⁹ See the discussion by L. E. Booher *J Am Med Assoc* 110 1920 (1938)

²⁶⁰ P. D. Cramm and D. M. Short *Am J Physiol* 118 477 (1937)

²⁶¹ H. R. Guilbert and G. E. Hart *J Nutrition* 10 409 (1935) H. R. Guilbert R. F. Miller and F. H. Hughes *Ibid* 13 543 (1937)

²⁶² H. Møllgaard *Biedermanns Zentr (B Tierernähr)* 10 214 (1938)

²⁶³ P. H. Phillips and G. Bohstedt *J Nutrition* 15 309 (1938)

²⁶⁴ R. Kuha and H. Brockmann *Alin Wochschr* 12 972 (1933)

²⁶⁵ P. Swomalainen *Skand Arch Physiol* 83 104 (1939)

²⁶⁶ J. T. Irving and M. B. Richards *Nature* 144 908 (1939)

²⁶⁷ Technical Commission for the Study of Nutrition Health Organization of the League of Nations *Geneva Bulletin* 7 April 1939

²⁶⁸ R. M. Sherwood and G. S. Frapa *Texas Agr Exptl Station Bull* M528 Sept 1936

²⁶⁹ J. K. Williams C. E. Lampman and D. W. Bolin *Poultry Sci* 18 268 (1939)

VITAMIN B₁—
THIAMIN

VITAMIN B₁—THIAMIN¹

1 Nomenclature and Survey

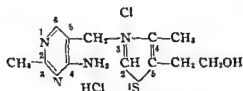
Names

- Thiamin American nomenclature ²
- Aneurin European nomenclature ³
- Oryzamin Japanese nomenclature ⁴
- Torulin Historical name ⁵
- Polynuramin Historical name ⁶
- Vitamin F Abandoned term ⁷
- The antineuritic anti beriberi vitamin

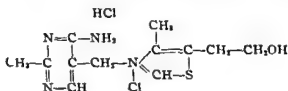
Chemical name

4 Methyl 5 β hydroxy ethyl N { [2 methyl 4 amino pyrimidyl (5)] methyl }
thiazolium chloride hydrochloride

Structure *



Some authors prefer to write the formula of vitamin B₁ as follows



No agreement exists as to the manner of writing the pyrimidine nucleus except in purine derivatives. The preferred form is the hexagonal to which the eye is accustomed and which best illustrates the conversion of vitamin B₁ to thiochrome

¹ R. R. Williams and T. D. Spies *Vitamin B* New York 1938

² Council on Pharmacy and Chemistry *J. Am. Med. Assoc.* 109 957 (1937)

B. C. P. Jansen *Nature* 135 767 (1935)

³ U. Suzuki, T. Shimomura and S. Odake *Biochem. Z.* 43 89 (1911)

⁴ C. S. Ide, W. H. Evans, B. Moore, C. C. E. Simpson and A. Webster *Biochem. J.* 6 234 (1912)

⁵ R. I. Jou *Science* 68 480 (1928)

⁶ H. C. Sherman and J. H. A. Mayer *J. Biol. Chem.* 75 207 (1927)

VITAMIN B₁—THIAMIN¹

1 Nomenclature and Survey

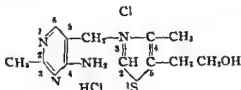
Names

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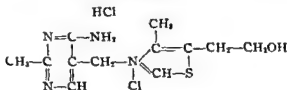
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¹ R. R. Williams and T. D. Spies *Vitamin B*, New York, 1934

² Council on Pharmacy and Chemistry *J. Am. Med. Assoc.* 109:95 (1937)

³ R. C. P. Jansen *Nature* 135:267 (1935)

⁴ I. S. Suk, T. Shimamura, and S. Odake *Biochem. Z.* 43:89 (1911)

⁵ C. S. F. W. H. I. v. S. B. Moore, G. C. I. Simpson, and A. Webster *Biochem. J.* 6:234 (1912)

⁶ R. I. Jon *Science* 68:480 (1924)

⁷ H. C. Sherman and J. H. Aximayer *J. Biol. Chem.* 75:407 (1922)

Empirical Formula



Efficacy

1 g = 333 000 International Units

2 Chronology

- 1885 TAKAKI⁸ prevented the occurrence of beriberi in the Japanese navy by changing the dietary ration
- 1893-1897 EIJKMAN⁹ produced experimental polyneuritis (beriberi) in fowls by a diet consisting exclusively of polished rice and prevented the disease by dietary means
- 1912 FUNK¹⁰ cured rats with dietary polyneuritis by the administration of water extracts of rice bran
- 1926 JANSEN and DONATH¹¹ isolated the vitamin in crystalline form from rice bran
- 1931 WINDAUS TSCHESCHE RUKOPF LAQUER and SCHULTZ¹² isolated the pure vitamin B₁ from yeast and established its empirical formula
- 1936 WILLIAMS and independently GREWE elucidated the chemical structure of vitamin B₁. In the same year the synthesis of this vitamin was accomplished by WILLIAMS and CLINK and by ANDERSAG and WESTPHAL
- 1937 LOHMANN and SCHUSTER isolated crystalline cocarboxylase from yeast and proved its constitution as the pyrophosphoric ester of vitamin B₁

3 Occurrence

Vitamin B₁ is present in many plants. Vegetables, fruits and nuts contain small amounts, ripe peas and beans are rich sources but vitamin B₁ is found most prevalently in outside bran coats of grains (rice) and in yeast (baker's yeast and brewer's yeast). Generally speaking, vitamin B₁ is present in high concentration in seeds that is in the nutritive material for the plant germ (see page 133). In most leaves the concentration of vitamin B₁ amounts to a constant value of about 25 International Units per 100 g, regardless of the botanical family.¹³

⁸ K Takaki *Ses + Kai Med J* August 1885 April 1886 6 73 (1887) *Lancet* 1906 I 136; 1451 1520

⁹ C Eijkman *Arch path Anat (Virchow's)* 148 523 (1897)

¹⁰ C Funk *J Physiol* 45 70 (1912)

¹¹ B C P Jansen and W F Donath *Mededeel Dienst Volksgezondheid Nederland Indië* 1926 (Pt I) 186 *Chem Weekblad* 23 201 (1926) *Koninkl Akad Wetenschappen Amsterdam Wisk Natu k Afd* 35 923 (1926)

¹² A Windaus R Tschesche H Rukopf F Laquer and F Schultz *J physiol Chem* 204 123 (1932) *Nachr Ges Wiss Göttingen Math physik Klasse* 1932 207 342

¹³ M Pyke *Biochem J* 34 330 (1940)

Some microorganisms are able to synthesize vitamin B₁. For example, certain bacteria exist in the intestinal tract of rats¹⁴ (especially in the colon) and of nursing children (especially in the great gut) that synthesize vitamin B₁ which is then found in the feces. Bacteria which produce vitamin B₁ also live in the rumen of cattle, sheep, etc.¹⁵

Vitamin B₁ is widely distributed in the animal organism in different organs (liver, kidney) and in muscles (especially in the heart).¹⁶ The actual amount is very small and varies greatly in different species. Pork muscles, for example, contain about eight times as much vitamin B₁ as beef muscles.¹⁷ Storage of large amounts does not occur in the animal organism.

Vitamin B₁ occurs in nature as the free compound or in the form of its salts as vitamin B₁ protein complex¹⁸ as vitamin B₁ pyrophosphoric acid ester (cocarboxylase) and as vitamin B₁ phosphorus protein complex. It is suspected that other vitamin B₁ containing compounds, for example, the monophosphate and other esters exist in tissues. The relative amounts of these forms vary considerably in different sources. Milk contains predominantly the free vitamin and the vitamin protein complex.¹⁹ The first colostrum contains practically no protein complexes. In skeletal and in heart muscle the amount of the free vitamin is greater than the phosphorylated compound, whereas in brain and liver the cocarboxylase (and its protein complex) occurs predominantly.²⁰

4 Isolation

Since vitamin B₁ is water soluble it is extracted, for example from rice polish or yeast with cold water which is brought to pH 4.5 by the addition of mineral acid. From the aqueous solution the vitamin is adsorbed on activated fuller's earth or charcoal at pH 6.5.²¹ Riboflavin which is also present in the water extract, is not adsorbed under these conditions.²² The vitamin is then extracted from the charcoal with dilute acids or from the fuller's earth with dilute alkali. By these methods however a con-

¹ J. Hyg. 27, 70 (1927)

² S. I. B. Chd. I. H. E. Honeywell, R. A. Dutcher and M. H. Knutsen, *J. Biol. Chem.* 30, 231 (1928)

³ H. G. K. W. Steubink, *Arch. Néerland. physiol.* 17, 60 (1932), 19, 116 (1932)

⁴ C. A. Elvehjem, W. C. Sherman and A. Arnold, *J. Biol. Chem.* 109, XXIV (1935), R. Hoagland, *J. Ag. Research* 83, 431 (1929)

⁵ J. Houston and S. K. Kon, *Nature* 143, 558 (1933)

⁶ J. Houston, S. K. Kon and S. V. Thompson, *J. Soc. Chem. Ind.* 58, 651 (1939), J. Houston and S. K. Kon, *Nature* 143, 558 (1939)

⁷ S. Ochoa and R. A. Peters, *Biochem. J.* 32, 1501 (1938)

⁸ H. W. Kinnorsley, J. R. O'Brien and R. A. Peters, *ibid.* 27, 232 (1933)

⁹ S. Ohdake, *J. Agr. Chem. Soc. Japan* 10, 409 (1934), R. Williams, R. R. Waterman and J. L. Keresztesy, *J. Am. Chem. Soc.* 56, 1187 (1934)

siderable amount of vitamin is lost. If, however, quinine salt solutions or salts of other organic bases are used, about five times as much vitamin is obtained²² (approximately 90% of the vitamin B₁ originally present)

Impurities can be precipitated by barium hydroxide²³ benzoyl chloride in the presence of an excess of sodium bicarbonate²⁴ silver nitrate in acid solution²⁵ and by many other reagents. The vitamin itself is precipitated by silver nitrate at pH 7.5²⁶ phosphotungstic acid at pH 4.5–5.5²⁷ silicotungstic acid²⁸ benzene sulfo chloride²⁹ picrolonic acid³⁰ Rufian acid³¹ Reinecke acid³² gold chloride in aqueous solution³³ platinum chloride in alcoholic solution³⁴ and mercuric chloride³⁵ in the presence of sodium acetate or carbonate³⁶ (but not by mercuric sulfate in acid solution³⁷) etc.

After the elution of the vitamin from the adsorbent, a combination of different precipitations is carried out. The vitamin B₁ is thus obtained as the hydrochloride. Four hundred and fifty pounds of rice polish or 2000 lbs of yeast yield about 1 g of the vitamin. For practical isolation procedures see page 118.

Vitamin B₁ and riboflavin, which usually occur together, are separated by precipitation of the riboflavin from an aqueous neutral or slightly alkaline solution with lead acetate, whereby vitamin B₁ remains in solution³⁸ Another method is a fractional adsorption first on charcoal at pH 4–5, which adsorbs all the riboflavin, and then on fuller's earth which adsorbs all the vitamin B₁.³⁹ On the other hand, vitamin B₁ may first be adsorbed from solutions of pH 3 on silica gel⁴⁰ or fuller's earth⁴¹. The riboflavin remains in solution and can be recovered from the filtrate.

²² A Seidell *J Biol Chem* 82 633 (1929)

²³ B C P Jansen and W F Donath *Mededeel Dienst Volksgezondheid Nederland Indië* 1926 (Pt I) 186 *Chem Weekblad* 23 201 (1926) *Koninkl Akad Wetenschappen Amsterdam Wisk Na* *turk Afd* 35 923 (1926)

²⁴ H W Kinnnersley J R O'Brien and R A Peters *Biochem J* 27 93 (1933)

²⁵ H W Kinnnersley and R A Peters *Ibid* 24 1856 (1930)

²⁶ B C P Jansen J P Wibaut P J Hubers and P W Wiardi *Rec trav chim* 52 366 (1933)

²⁷ B C P Jansen *Ibid* 48 984 (1929)

²⁸ A C van Veen *Z physiol Chem* 208 125 (1937)

²⁹ A Windaus R Tschesche H Ruhkopf F Laquer and F Seibitz *J physiol Chem* 204 123 (1932) *Nachr Ges Wiss Göttingen Math physik Klasse* 1932 342

³⁰ S Osdake *Proc Imp Acad (Tokyo)* 7 102 (1931) 8 179 (1932) 10 45 (1934)

³¹ A Seidell and M I Smith *J Am Chem Soc* 55 3350 (1933)

³² B C Guha *Biochem J* 25 931 (1931)

³³ B C Guha and J C Drummond *Ibid* 23 880 (1929)

³⁴ B C P Jansen J P Wibaut P J Hubers and P W Wiardi *Rec trav chim* 52 366 (1933)

³⁵ B C P Jansen H W Kinnnersley R A Peters and V Reader *Biochem J* 24 1824 (1930)

³⁶ H Chick and M H Roscoe *Ibid* 23 804 (1929)

³⁷ J I Rosedale *Ibid* 21 1266 (1927)

³⁸ R D Creene and A Black *J Am Chem Soc* 59 1395 (1935)

³⁹ P A Levene *J Biol Chem* 79 465 (1928) *Science* 71 668 (1930)

⁴⁰ W D Salmon N B Carrant and I M Hays *J Biol Chem* 80 91 (1928)

5 Properties

Vitamin B₁ hydrochloride is soluble in water (1 g in 1 cc) and alcohols (1 g in 100 cc of 95% alcohol or in 315 cc absolute alcohol, or in 18 cc of glycerin), but insoluble in ether, chloroform, benzene and acetone²⁴ It is optically inactive²⁶ Vitamin B₁ hydrochloride crystallizes from alcoholic aqueous solutions as the hemihydrate (colorless monoclinic needles), melting at 248–250°⁴² The bromide hydrobromide hemihydrate occurs as rosettes of needles that melt at 229–231°

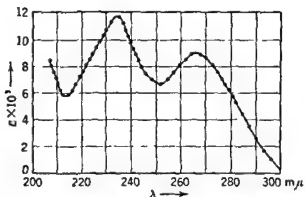


Fig. 6.—Absorption spectrum of vitamin B₁ (O Wintersteiner, R. R. Williams and A. E. Ruehle)

The melting points of various other salts are as follows: sulfate m p 203° and 276–278°⁴³; nitrate m p 164–165°⁴³; picrolonate (dimorphous) m p 165° or 229°, picrate m p 208°; gold salt m p 189°; rufinate m p 291°⁴⁴. All melting points are somewhat uncertain because of attendant decomposition. The free vitamin base may be obtained from the chloride in amorphous form by treatment with silver oxide. On standing in air of average humidity vitamin B₁ crystals absorb humidity in an amount of one mol. Vitamin B₁ crystals and solutions have a slight yeast like or nutty odor.

The ultraviolet absorption spectrum of the vitamin hydrochloride shows two bands at a pH of 7 (or greater) at 235 mμ and 267 mμ, respectively⁴⁵

²⁴ J. K. Choe, R. R. Williams and J. Finkelstein, *J. Am. Chem. Soc.* 59, 1052 (1937); R. R. Williams and J. K. Choe, *Ibid.* 59, 216 (1937).

⁴³ W. Kinnerly, J. R. O'Brien and R. A. Peter, *Biochem. J.* 29, 701 (1935).

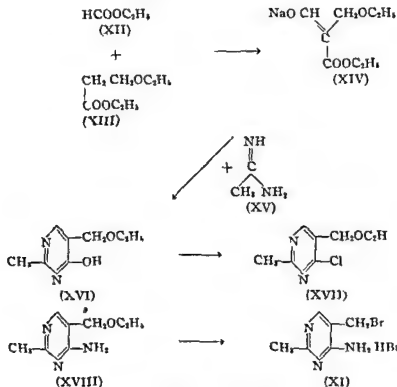
⁴⁴ R. R. Williams, *Ergeb. Vitaminforsch.* 1, 217 (1938).

⁴⁵ O. Wintersteiner, R. R. Williams and A. E. Ruehle, *J. Am. Chem. Soc.* 57, 517 (1935).

synthesis of vitamin B₁ (see page 116) Grewe converted the 5 amino methyl compound (VII) (see above) into the corresponding 5 bromo methyl compound (XI)⁸⁶



Chas. Williams and Finkelstein obtained the compound (XI) in the following way⁸⁷ By condensing ethyl formate (XII) and β ethoxy ethyl



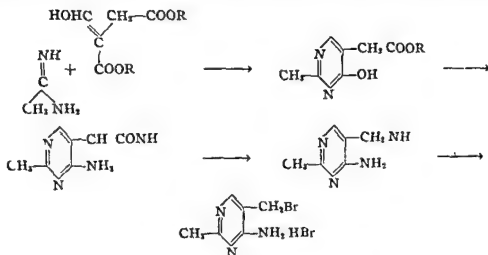
propionate (XIII) with sodium sodium formyl β ethoxy ethyl propionate was obtained (XIV) which by condensation with acetamide hydrochloride (XV) yielded 2 methyl-4 hydroxy 5 ethoxy methyl pyrimidine (XVI) The hydroxyl group in 4 position was converted into the chloride (XVII) by phosphorus-oxychloride and finally into an amino group (XVIII) by ammonia in alcohol By the action of hydrobromic acid the

⁸⁶ See also T. Imai and K. Makino *Z. Phys. Chem.* 252 76 (1938)

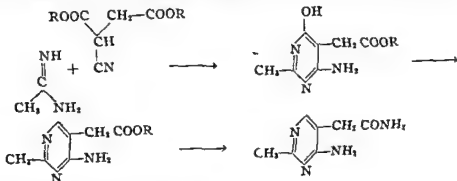
⁸⁷ J. K. Clue, R. R. Williams and J. Finkelstein *J. Am. Chem. Soc.* 59 10 2 (1937)

ethoxy group in the methyl in 5 position was replaced by bromine, yielding the 2 methyl 4 amino 5 bromo methyl pyrimidine hydrobromide (XI)

Andersag and Westphal have chosen another route ⁶⁸ By condensation of acetamidine with formyl ethyl succinate, 2 methyl 4 oxypyrimidine 5 ethyl acetate is formed. The hydroxyl group is replaced by chlorine and the chloro compound on reaction with liquid ammonia yields 2 methyl-4 amino pyrimidine 5 acetamide. By a Hoffmann degradation 2 methyl 4 amino 5 methyl amino pyrimidine is obtained nearly quantitatively. By the action of nitrous acid on the diamine, only the aliphatic amino group is converted into the corresponding alcohol. Hydrobromic acid treatment yields finally the 2 methyl-4 amino 5 bromo methyl pyrimidine hydrobromide



A modification of this method consists in the condensation⁶⁹ of acetamidine with ethyl cyano succinate, yielding 2 methyl-4 amino 6-oxypyrimidine 5 ethyl acetate. The hydroxyl group in 6 position is converted into a



⁶⁸ H. Andersag and K. Westphal *Ber* 70 2035 (1937)

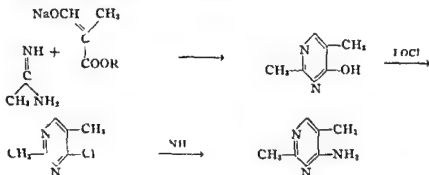
⁶⁹ H. Andersag and K. Westphal *G F* 671 78

chlorine group and the chlorine is eliminated by zinc dust yielding the 2-methyl-4-amino-pyrimidine-5-ethyl acetate. Ammonia treatment yields then the 5-acetamide compound of the previously mentioned synthesis.

As has been mentioned before the sulfite cleavage product of vitamin B₁ that contains the pyrimidine ring has the formula (II). This acid was synthesized by Grewe⁷⁰ from the previously described 2-methyl-4-amino-5-bromo-methyl-pyrimidine by heating with an acid bisulfite solution.



By reduction with sodium in liquid ammonia the amino-sulfonic acid (II) gives a base⁷¹ C₆H₇N₃, the picrate of which proved to be identical with that of 2,5-dimethyl-4-amino-pyrimidine. The latter compound was synthesized⁷² by condensing acetamidine and sodium formyl-ethyl-propionate transforming the obtained 2,5-dimethyl-4-hydroxy-pyrimidine into the corresponding 4-chloro compound and exchanging the chlorine with ammonia.



The oxy-sulfonic acid, 2-methyl-4-hydroxypyrimidine-5-methyl-sulfonic acid, mentioned before as the reaction product of hydrochloric acid with the amino-sulfonic acid (II) has been obtained by reacting the synthetically obtained 2-methyl-4-hydroxy-5-ethoxy-methyl-pyrimidine with sodium sulfite⁷³.

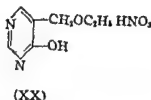
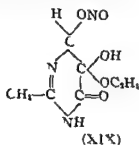


⁷⁰ R. Grewe *Z. phys. Chem.* 242 89 (1936).

⁷¹ J. A. Cline, R. R. Williams, A. F. Ruehle and R. I. Waterman *J. Am. Chem. Soc.* 59 370 (1937).

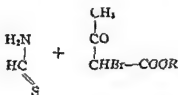
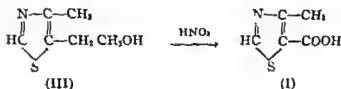
⁷² R. R. Williams, A. F. Ruehle and J. I.inkel *ibid.* 59 376 (1937).

By mild oxidation of vitamin B₁ with nitric acid another pyrimidine derivative is obtained. It is an ethyl ester of the formula $C_7H_{11}N_2O_5$ and may have the constitution of (XIX) according to Grewe^{73, 74} or of (XX) according to Williams⁷⁵



(b) The Thiazole Part

The other reaction product of the sulfite cleavage of vitamin B₁ is the base C_6H_7ONS (III). The sulfur in this compound is, in differentiation to the sulfur in the pyrimidine part of the cleavage product, in the same form as in the vitamin. By oxidation of the base with nitric acid the side chain is degraded, yielding the acid (I), which is also obtained by direct



nitric acid oxidation of the vitamin,⁷⁶ and is identical with the synthetically^{77, 78} prepared 4-methylthiazole-5-carboxylic acid.^{79, 80} This syn-

⁷³ A. Windaus, R. Tschesche and R. Grewe, *Z. physiol. Chem.* 237, 98 (1933).

⁷⁴ R. Grewe, *Ibid.* 242, 89 (1936).

⁷⁵ R. R. Williams, *J. Am. Chem. Soc.* 55, 1063 (1933).

⁷⁶ A. Windaus, R. Tschesche and R. Grewe, *Z. physiol. Chem.* 228, 27 (1928).

⁷⁷ M. Wohmann, *Ann.* 259, 299 (1890).

⁷⁸ M. L. Tomlinson, *J. Chem. Soc.* 1935, 1030.

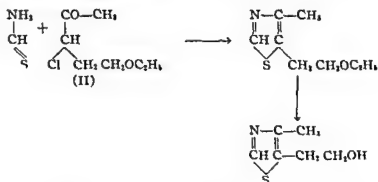
⁷⁹ H. T. Clarke and S. Gurin, *J. Am. Chem. Soc.* 57, 1676 (1935).

⁸⁰ K. R. Buchman, *Ibid.* 58, 1803 (1936).

thesis can be carried out by condensing thioformamide with α bromo aceto acetic ester

The presence of a thiazole ring in vitamin B₁ was indicated by ultra violet absorption studies, by the stability against nitric acid and the loss of sulfur by the action of alkaline plumbite.

The thiazole part of the sulfite cleavage of vitamin B₁ is an alcohol and has one carbon atom more than the acid obtained by its nitric acid oxidation. The alcohol is 4 methyl 5 β hydroxy ethyl thiazole (III). The presence of the hydroxyl group was proved⁸¹ by the formation of a chloride and a *p* nitrobenzoate. Since this benzoate was found to be basic the presence of a tertiary nitrogen was suspected. This was further proved by the formation of a quaternary salt with methyl iodide. The primary nature of the alcoholic group was indicated by the result of its nitric acid oxidation by the optical inactivity of the alcohol and the failure of the iodoform test. These indications were substantiated by synthesis. The alcohol has been synthesized⁸² by condensation of thioformamide with the chloro ketone (II) (methyl α chloro γ ethoxy propyl ketone) and hydrolysis to the corresponding alcohol.



A variety of modifications of this method are also possible. Todd and co workers⁸³⁻⁸⁵ used instead of the chloro ethyl ether (II) the corresponding chloro ethyl acetate the ester group of which can be saponified with more facility than the ether group. Buchman⁸⁶ simplified the preparation by condensing acetoacetic acid ester with ethylene oxide to α acetyl butyrolactone chlorinating the lactone in α position with sulfur

⁸¹ F. R. Buchman, R. R. Williams and J. C. Kerestesy, *J. Am. Chem. Soc.* 57, 1840 (1935).

⁸² H. T. Clarke and S. G. Finn, *Ibid.* 57, 1870 (1935).

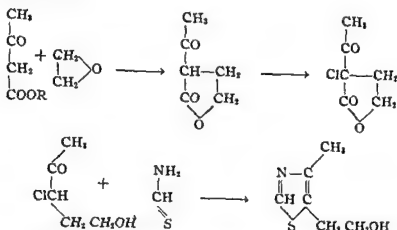
⁸³ G. Barger, F. Bergel and A. R. Todd, *Nature* 136, 759 (1935); *Be.* 68, 22, 7 (1935).

⁸⁴ A. R. Todd, F. Bergel and Karimullah, *Ber.* 69, 217 (1936).

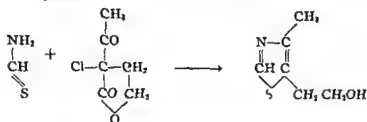
⁸⁵ A. R. Todd, F. Bergel, Karimullah and R. K. He, *J. Chem. Soc.* 1937, 761. A. R. Todd, F. Bergel and A. Jacob, *Ibid.* 1936, 1555.

⁸⁶ F. R. Buchman, *J. Am. Chem. Soc.* 58, 1803 (1936).

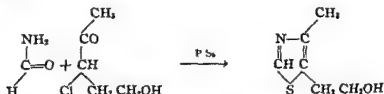
chloride and obtaining by hydrolysis γ chloro γ acetyl propyl alcohol, which by condensing with thioformamide yielded the 4 methyl 5 β hydroxy ethyl thiazole



A further simplification was made by Wenz,⁸⁷ who found that the α chloro α acetyl butyro lactone can be condensed with thioformamide directly to the thiazole compound



Another modification, by Hromatka,⁸⁸ was the condensation of formamide with γ chloro γ acetyl propyl alcohol in the presence of phosphorus pentasulfide



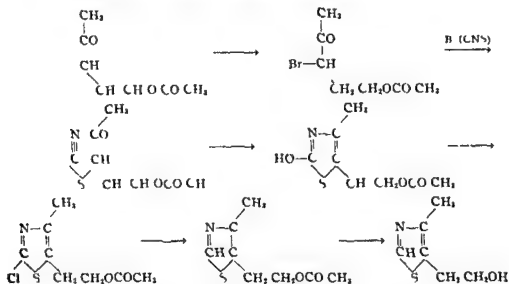
A different synthesis was announced by Andersag and Westphal⁸⁹ γ aceto propyl acetate is brominated and the bromo compound condensed with barium thiocyanate, forming γ thiocyno γ aceto propyl acetate. This rearranges in acid solution to the isomeric 2 hydroxy-4 methyl 5

⁸⁷ A. Wenz G. P. 664 789

⁸⁸ O. Hromatka U. S. P. 2 160 867

⁸⁹ H. Andersag and K. Westphal Ber. 70 2035 (1937)

acetoxy ethyl thiazole The hydroxyl group in 2 position is converted into chlorine by phosphorus oxychloride and the chloride by means of zinc and acetic acid is transformed into 4 methyl 5 acetoxy ethyl thiazole which by saponification yields 4 methyl 5 β hydroxy ethyl thiazole



(c) The Connection of the Pyrimidine and the Thiazole Part

The pyrimidine compound and the thiazole compound are connected in the vitamin B₁ molecule by a methylene bridge. The presence of the methylene group was suspected by the ultraviolet absorption of the vitamin⁹⁰ and was proved by synthesis. The pyrimidine portion is connected with the carbon atom to which the sulfonic acid group is added by the sulfite cleavage process. The thiazole portion is connected with the tertiary nitrogen atom, since in the vitamin itself the nitrogen is quaternary. This has been shown by the already mentioned addition of methyl iodide to the thiazole compound and by potentiometric titrations of the quaternary methyl iodide compound and of the vitamin hydrochloride⁹¹⁻⁹⁴ (see Fig. 7).

Williams states⁹⁵ On titrating thiamin hydrochloride with alkali, we find a sharp rise when 1 mol is reached corresponding to the formation of the neutral or mono acid salt but there is no further break until a total of

⁹⁰ K. Makino and T. Im: *Z. physiol. Chem.* 239: 1 (1936).

⁹¹ H. T. Clarke and S. Curran: *J. Am. Chem. Soc.* 57: 1876 (1935).

⁹² T. W. Birch and L. J. Harris: *Nature* 135: 654 (1935).

⁹³ R. C. G. Moggridge and A. G. Ogsten: *Biochem. J.* 29: 866 (1935). A. G. Ogsten and R. A. Peters: *ibid.* 30: 736 (1936).

⁹⁴ R. R. Williams and A. E. Ruehle: *J. Am. Chem. Soc.* 57: 1836 (1935).

⁹⁵ R. R. Williams and T. D. Spies: *Isolation of Vitamin B₁*, New York, 1938, p. 163.

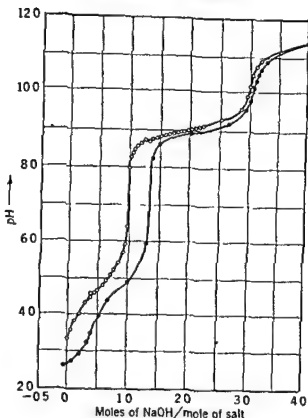
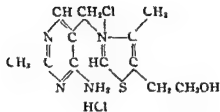


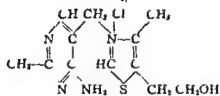
Fig 7 Titration of vitamin B₁ chloride with NaOH (O—O) and back titration with HCl (●—●) (R R Williams and A E Ruehle)

3 mols has been added. Apparently the addition of 1 mol of alkali liberates the monochloride (*b*). Further addition of alkali begins to liberate the strong quaternary base (*c*) which goes over rapidly into the neutral pseudo base or carbinol (*d*). This does not occur instantaneously, as one can see during titration by the momentary rise and subsequent slow fall of pH after each addition of alkali. Such process of the migration of hydroxyl is well known in cyclic quaternary bases and in effect uses up hydroxyl ions. Nor does the pH rise even when 2 mols have been added because quaternary thiazoles such as this undergo ring opening in alkali solution, forming an acidic sulfhydryl group⁶⁶. Only after this is neutralized with a third mol of alkali to form (*e*) does the alkalinity rise sharply, indicating free NaOH. The reverse arrows indicate reversal of the sequence and the regeneration of the vitamin upon back titration. If the solution stands in alkaline condition for some time, there is some permanent destruction of the vitamin.

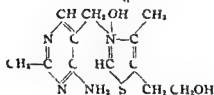
⁶⁶ W H Mills I M Clark and J A Aeschlimann *J Am Chem Soc* 123 933 (1921)



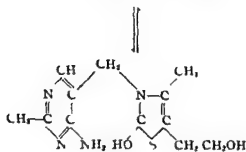
(a)



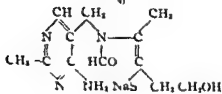
(b)



(c)

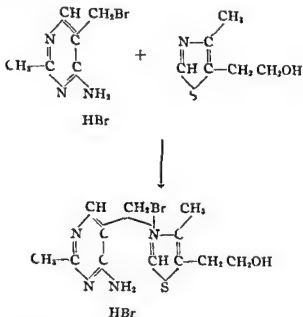


(d)



(e)

The final proof for the connection of both fragments is given by the total synthesis of vitamin B₁. 2 Methyl 4 amino 5 bromo methyl pyrimidine hydrobromide upon condensation with 4 methyl 5 β oxy ethyl thiazole yields the vitamin B₁ bromide hydrobromide^{97 98 99}



The bromide hydrobromide can be converted into the chloride hydrochloride either by shaking with silver chloride⁹⁸ or by preparing the sparingly soluble picrate followed by digestion with 10% hydrochloric acid⁹⁹

An improvement¹⁰⁰ in this method consists of condensing the hydrochloride of the 5-hydroxymethyl pyrimidine compound with the hydrochloride of the thiazole compound, giving the vitamin chloride hydrochloride directly

An entirely different method of synthesizing vitamin B₁ has been effected^{101 102} by condensing 2-methyl-4-amino-5-thioformamido-methyl pyrimidine, which can be obtained from the 5-amino-methyl compound by condensation with potassium dithioformate or with ethyl formate in the presence of phosphorus pentasulfide with γ-chloro (or bromo) γ-aceto-propyl alcohol (or acetate or benzoate)

⁹⁷ R. R. Williams and J. K. Cline *J. Am. Chem. Soc.* **58**, 1504 (1936)

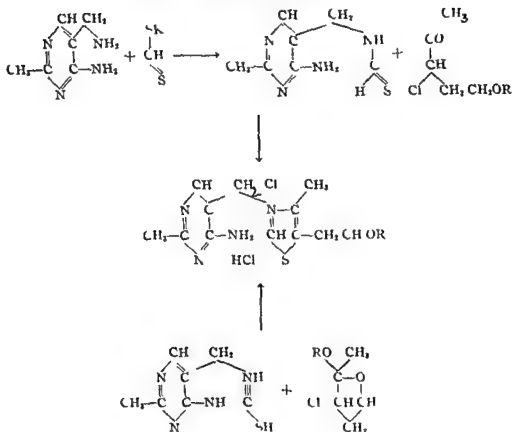
⁹⁸ J. K. Cline, R. R. Williams and J. Finkelstein *Ibid.* **59**, 1052 (1937)

⁹⁹ H. Andersag and K. Westphal *Ber.* **70**, 2035 (1937)

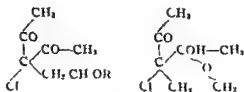
¹⁰⁰ O. Zima *G. P.* 669, 187

¹⁰¹ A. R. Todd and F. Bergel *J. Chem. Soc.* 1937, 364

¹⁰² H. Andersag and K. Westphal *Ber.* **70**, 2035 (1937)



Instead of the γ halogen γ aceto propyl alcohol also $\gamma\gamma$ diaceto γ halogeno (or mercapto) propyl alcohol or its inner hemiacetale might be used¹⁰³

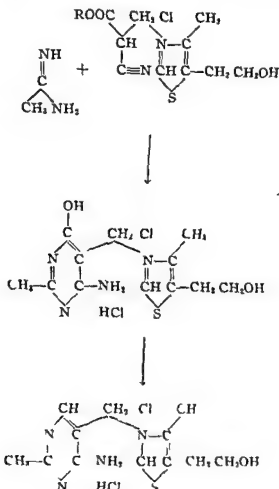


A modification of this method which is said to give much better yields¹⁰⁴ consists in the condensation of 2 methyl-4 amino 5 thioform amido methyl pyrimidine with 2 methyl 2 alkoxy 3 chloro tetrahydrofuran which can be obtained from the previously described α chloro α aceto butyro-lactone and a primary alcohol in the presence of sulfuric acid

¹⁰³ T. M. I. Kawa and M. Oht. U. S. 2 184 7 0 Ind. an. 55809

¹⁰⁴ M. Klugefuss. U. S. P. 1 274 46 G. P. 676 980 I. P. 831 110

A different method of synthesizing vitamin B₁ should finally be mentioned¹⁰⁶ It consists in building the pyrimidine nucleus on the thiazole nucleus and is best described by the following series of formulas



7 Industrial Methods of Preparation

Vitamin B₁ is manufactured today by total synthesis according to the methods described before. The isolation from natural sources which is more expensive is however, still carried out technically. The latter method has been worked out systematically¹⁰⁶ The vitamin is adsorbed on fuller's earth or synthetic zeolites^{107 108 109} extracted with acid salts

¹⁰⁶ H. Andersug and K. Westphal *F. P.* 81f 432

¹⁰⁶ R. D. Greene and A. Black *J. Am. Chem. Soc.* 59 139a (1937)

¹⁰⁷ L. R. Cerecedo and D. J. Hennessy *Ibid.* 59 161* (1937)

¹⁰⁸ J. R. Cerecedo and P. J. K. *ibid.* 59 1119 (1937)

¹⁰⁹ J. R. Cerecedo and J. J. Thon *ibid.* 59 1621 (1937)

of organic nitrogen containing bases such as pyridine or quinine the adventitious matter removed by benzylation in soda¹ alkaline solution and chloroform extraction, followed by precipitation of the vitamin with silver nitrate barium hydroxide and phosphotungstic acid Vitamin B₁ is finally recrystallized from acidified organic solvents, such as combinations of phenol and butyl alcohol or HCl alcohol From one ton of rice polishings 5-10 g of vitamin B₁¹¹⁰ can be obtained by this method

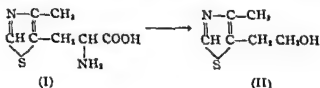
Commercial vitamin B₁ contains about 4% water which can be removed by drying at 100° C or by standing in a vacuum over sulfuric acid Elixirs of thiamin chloride are marketed which contain a wine base Since tannic acid precipitates vitamin B₁ the wine is completely freed from tannic acid by the use of freshly prepared casein or completely defatted and dealbuminized milk¹¹¹

The preparation of the vitamin B₁ pyrophosphate, which will be described later is also commercially important

8 Biogenesis

The natural precursors of vitamin B₁ and the reactions leading to the synthesis of vitamin B₁ in the plant organism are unknown The hypothesis has been advanced^{112 113} that the particular thiazole part of the vitamin B₁ molecule might arise during yeast fermentation from α amino β (4 methyl thiazole 5) propionic acid (I), which could have been built up from methionine acetaldehyde and ammonia, by a reaction analogous to the formation of fusel oil in alcoholic fermentation Yeast has been found to be able to convert this amino propionic acid derivative of thiazole (I) into the 4 methyl 5 (β hydroxy ethyl) thiazole (II) of vitamin B₁

(For the influence of light and chlorophyll upon the vitamin B₁ synthesis and for the site of the synthesis in higher plants see page 133)



¹¹⁰ R R Williams R T Waterman and J C Keresztesy *J Am Chem Soc* 56 1187 (1934)

¹¹¹ I Grengard *J Am Pharm Assoc* 1 230 (1940)

¹¹² C R Harrington and R C G Moggridge *Biochem J* 34 682 (1940) *J Chem Soc* 1939 443

¹¹³ J Boner and F R Buchman *Proc Natl Acad Sci U S A* 24 431 (1939)

9 Thiochrome

By oxidation, vitamin B₁ is converted into thiochrome a yellow substance of intense blue fluorescence¹¹⁴ This conversion occurs also when alcoholic solutions of the vitamin stand at room temperature for several months¹¹⁵ Very little thiochrome is formed at pH 2, but it is produced more rapidly as the pH approaches 7 Thiochrome can be obtained from vitamin B₁ by oxidation with permanganate or manganese oxides at pH 7,¹¹⁶ by oxidation of alkaline solutions of vitamin B₁ with potassium ferricyanide,¹¹⁷ by hydrogen peroxide, selenium dioxide, etc¹¹⁸ The same substance has been isolated from yeast by Kuhn and co workers¹¹⁹ who proposed the name thiochrome The compound melts at 227–228° and exhibits absorption maxima at 358 and 375 mμ (see Fig 8) The isolation of thio

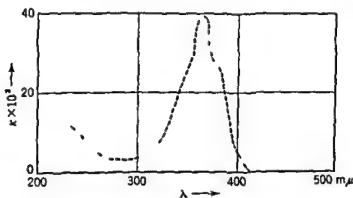


Fig 8—Absorption spectrum of thiochrome (R Kuhn and H Vetter)

chrome has been carried out by adsorption on acid silicates, followed by elution and precipitation with silver nitrate From 8100 lbs yeast, about 250 mg thiochrome are obtained Thiochrome probably does not occur as such in yeast, but is formed when the yeast is worked up Sodium hydrosulfite reduces thiochrome in neutral or alkaline solutions to a leuco compound which does not fluoresce By air oxidation thiochrome is regenerated In alkaline solutions thiochrome is sensitive to light and the

¹¹⁴ R A Peters *Nature* 135 107 (1935)

¹¹⁵ H W Kinnorsley J R O'Brien and R A Peters *Biochem J* 29 701 (1935)

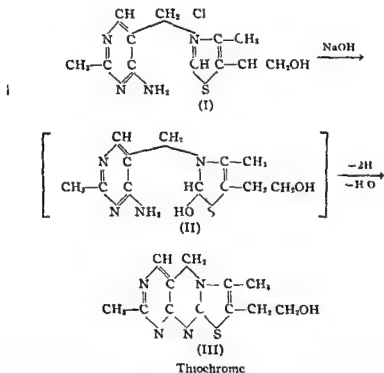
¹¹⁶ H W Kinnorsley J R O'Brien and R A Peters *Ibid* 29 2369 (1935)

¹¹⁷ G Barger F Bergel and A R Todd *Nature* 136 259 (1935) *Ber* 68 2757 (1935)

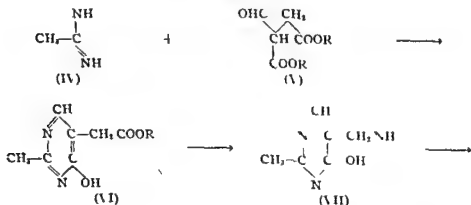
¹¹⁸ R Kuhn and H Vetter *Ber* 68 2375 (1935)

¹¹⁹ R Kuhn T Wagner Jauregg F W von Klaveren and H Vetter *Z physiol Chem* 234 196 (1935)

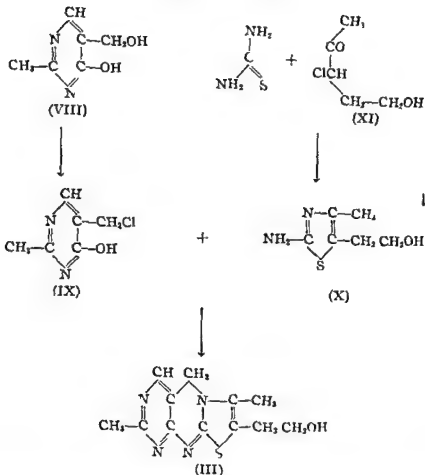
fluorescence disappears irreversibly. The mechanism of the dehydrogenation of vitamin B₁ to thiochrome is not known with certainty. It is however, very probable that at first an intermediate is formed of the following formula (II)



Thiochrome has been prepared synthetically in the following manner ¹²⁰



¹²⁰ A. R. Todd & Bergel H. L. Frankel, Conant and A. J. Job, *J. Chem. Soc.* 1936 11401



Condensation of acetamidine (IV) with formyl succinate (V) yielded 4 hydroxy 2 methyl pyrimidine 5 ethyl acetate (VI) from which by Curtius degradation 4 hydroxy 5 amino methyl 2 methyl pyrimidine (VII) was obtained. Replacement of the amino group by hydroxyl was effected by means of nitrous acid and the resulting 4 hydroxy 5 hydroxy methyl 2 methyl pyrimidine (VIII) on boiling with phosphorus chloride yielded the chloro compound (IX). 2 Amino 4 methyl 5 β hydroxy ethyl thiazole (X) was obtained by condensing methyl α chloro γ hydroxy propyl ketone (XI) with thiourea. The pyrimidine and the thiazole compound form thiochrome upon condensation.

Compounds with the same ring skeleton as thiochrome are called quinochromes.

10 Vitamin B₁-Pyrophosphate

The biological action of vitamin B₁ is partly or mainly due to the action of its pyrophosphoric acid ester. Vitamin B₁ apparently acts as an ac

By enzymatic dephosphorylation with prostata phosphatase or with a phosphatase which is liberated from yeast cells during drying,¹²⁸ free vitamin B₁ is obtained. Only one molecule of phosphonic acid is hydrolyzed by the action of alkaline kidney phosphatase¹²⁹ ¹³⁰ or by acid hydrolysis. The second mol of phosphoric acid is much more difficult to hydrolyze. Neither the monophosphoric acid obtained nor vitamin B₁ shows cocarboxylase action.

The chemical reactions of cocarboxylase resemble closely those of the free vitamin B₁. By slight oxidation, a blue fluorescent compound of the thiochrome type is obtained. By the action of sulfite the molecule is split as described for the vitamin itself, into the pyrimidine part C₄H₅O₃N₃S and the diphosphorylated thiazole compound.

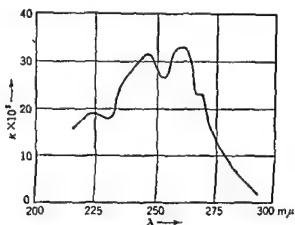


Fig 9—Absorption spectrum of vitamin B₁ pyrophosphate (cocarboxylase) (H Rudy in W Stepp *Ernährungslehre*)

Vitamin B₁ pyrophosphate in aqueous solution forms an inner salt connecting the amino group of the pyrimidine part with one of the hydroxyl groups of the phosphoric acid part. The absorption spectrum of vitamin B₁ pyrophosphate (see Fig 9) resembles closely that of the free vitamin.

The synthesis of cocarboxylase has been achieved by enzymatic and by chemical methods. Phosphatase¹³¹ of the duodenal mucosa of the pig, dried brewer's yeast and living yeast¹³² were successfully employed as enzymatic agents for the phosphorylation of vitamin B₁ in the presence of

¹²⁸ D Melnick and H Field *J Biol Chem* 127 531 (1939)

¹²⁹ K. Lohmann and P. Schuster *B. chem. Z.* 294 188 (1937)

¹³⁰ H. Tauber *J. Biol. Chem.* 123 499 (1938)

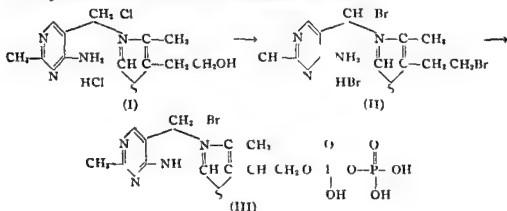
¹³¹ H. Lauter *Science* 86 180 (1937) *Enzymologia* 2 171 (1937) *J. Biol. Chem.* 123 499 (1938)

¹³² H. W. Kannersley and R. A. Peters *J. Soc. Chem. Ind.* 56 447 (1937) *Biochem. J.* 32 697 (1938)

M. A. Lipschitz, Van R. Potter and C. A. Elvehjem *B. chem. J.* 32 474 (1938)

hexose diphosphate and adenine triphosphate¹³³ Phosphoglyceric acid and phosphopyruvic¹³⁴ acid can also act as phosphate donor in the presence of catalytic amounts of adenylic acid or adenylyl pyrophosphate¹³⁵ Cocarboxylase is also enzymatically prepared from vitamin B₁ monophosphate which apparently is first hydrolyzed to the non phosphorylated vitamin¹³⁵ All these enzymatic processes occur only in the presence of the apocarboxylase the specific protein of the carboxylase and are stopped when the protein is partly or fully saturated^{134 135} Therefore no preparative use can be made of the enzymatic synthesis of cocarboxylase Cocarboxylase has also been obtained from vitamin B₁ by bacterial synthesis using *Propionibacterium pentosaceum*¹³⁶

Chemically, cocarboxylase was synthesized from vitamin B₁ in low yields by the action of phosphorus oxychloride¹³⁷ with better yields by condensation with sodium pyrophosphate in the presence of phosphoric acid¹³⁸ or by the use of pyrophosphoryl chloride¹³⁹ Another method consists in the conversion of vitamin B₁ (I) into the bromo vitamin (II) by the action of hydrobromic acid The bromo compound in turn is reacted with a solution of silver pyrophosphate in pyrophosphoric acid to yield the cocarboxylase (III)¹⁴⁰ For the purification of the synthesized cocarboxylase chloride a precipitation with phosphotungstic acid has been recommended and final crystallization is achieved from alcoholic HCl¹⁴¹



¹³³ H v Euler and R Vest *n Naturwissenschaften* 25 416 (1937)

¹³⁴ M A Lipton and C A Fiveshjem *Cold Spring Harbor Symposium on Quant Biol* 7 (1933) *Nature* 143 226 (1940)

¹³⁵ H Weil Malherbe *J Soc Chem Ind* 58 1071 (1939)

¹³⁶ M Silverman and C H Werkman *Proc Soc Exptl Biol Med* 40 369 (1939)

¹³⁷ K G Stern and J W Hofer *Enzymologia* 3 82 (1937)

¹³⁸ J Weijlard and H Tauber *J Am Chem Soc* 60 730 2763 (1938)

¹³⁹ A Lohmann quoted in C Oppenheimer and K G Stern *Biological Oxidation* New York 1939 p 207

¹⁴⁰ H Weil Malherbe *J Soc Chem Ind* 58 1071 (1939) *Biochem J* 34 980 (1940)

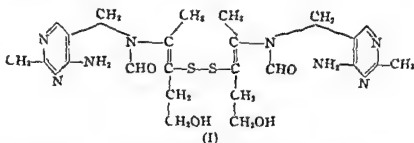
¹⁴¹ J Weijlard *J Am Chem Soc* 63 1160 (1941)

Cocarcboxylase as well as the vitamin monophosphate have the biological activity of the vitamin B₁ ¹⁴²

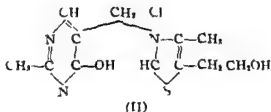
The chemistry of the apocarcboxylase, the protein part of the carboxylase is not known. The molecular weight is about 150,000 ¹⁴⁴

11 Specificity of the Vitamin B₁ Action

The vitamin action of vitamin B₁ seems to be connected with the specific structure of the molecule. The different vitamin salts, as the hydrochloride, hydrobromide, ¹⁴⁴ sulfate etc., and the pyrophosphoric acid ester, cocarcboxylase have a corresponding activity. The vitamin B₁ disulfide (I) is also as active as the vitamin



Structural alterations, however, cause disappearance of the vitamin action. Inactive are thiochrome ¹⁴⁵ ¹⁴⁶ the oxychlorothiamin ¹⁴⁷ (II) and the products obtained from the sulfite cleavage of the vitamin ¹⁴⁸ ¹⁴⁹. Dihydro



vitamin B₁ is also inactive, but the dihydro cocarcboxylase was found to be active ¹⁵⁰. Polyneuritis in pigeons may however, be cured ¹⁵¹ by 4 amino

¹⁴² K. Lohmann and P. Schuster *Biochem Z.* 294 188 (1937)

¹⁴³ C. Oppenheimer and K. G. Stern *Biological Oxidation* New York 1939 p 208

¹⁴⁴ R. R. Williams and J. K. Cline *J. Am. Chem. Soc.* 58 1804 (1936)

¹⁴⁵ G. Barger, F. Bergel and A. R. Todd *Nature* 135 59 (1935) *Ber.* 68 277 (1935)

¹⁴⁶ R. Kuhn and U. Vetter *Ber.* 68 2375 (1935)

¹⁴⁷ T. R. Buchman and R. R. Williams *J. Am. Chem. Soc.* 57 1751 (1935)

¹⁴⁸ R. R. Williams *Ibid.* 57 279 (1935)

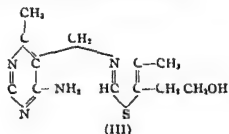
¹⁴⁹ R. R. Williams, R. E. Waterman, J. C. Keresztesy and T. R. Buchman *Ibid.* 57 337 (1935)

¹⁵⁰ C. Oppenheimer and K. G. Stern *Biological Oxidation* New York 1939 p 204

¹⁵¹ W. J. Robbins, M. A. Bartley, A. G. Hogan and I. R. Richardson *Proc. Natl. Acad. Sci. U. S.* 23

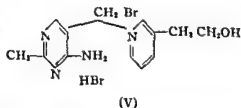
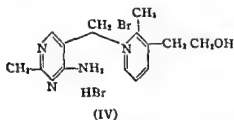
2 methyl 5 bromo methyl pyrimidine and 4 methyl 5 β hydroxy ethyl thiazole if given simultaneously (A synthesis of vitamin B₁ probably occurs from the intermediates)

A great number of compounds have been prepared synthetically¹⁵ with slight differences in the vitamin structure. Only one (III) has been found active and that one only in much larger doses¹⁵³



The pyrimidine ring, the thiazole ring and the methylene bridge between them, an unsubstituted amino group in 4 position of the pyrimidine ring,¹⁵⁴ the 5 hydroxy alkyl group¹⁵⁵ and a free 2 position in the thiazole nucleus are all necessary for the vitamin action. It is also probable that the nature of the substituents in position 6 influences the vitamin activity.¹⁵

So far as is known only one substance with vitamin B₁ properties occurs naturally. Growth action of *Phycomyces* is however also obtained by the 2 ethyl instead of the 2 methyl derivative¹⁵⁶. Hetero vitamin B₁ (IV) containing instead of the thiazole ring a pyridine ring has an activity of about $1/26$ of that of vitamin B₁.¹⁵⁷



The next lower homolog (V) devoid of the methyl group in the pyridine ring is only $1/10$ as active as the methylated product.¹⁵⁸

¹⁵³ F. Bergel and A. R. Todd, *J. Chem. Soc.* 1937, 1504.

¹⁵⁴ H. Andersag and K. Westphal, *Ber.* 70, 2035 (1937).

¹⁵⁵ F. Bergel and A. R. Todd, *J. Chem. Soc.* 140, 1504 (1933).

¹⁵⁶ D. Price and F. D. Pickel, *J. Am. Chem. Soc.* 63, 1067 (1941).

¹⁵⁷ W. J. Robbins and F. Kavanaugh, *Proc. Natl. Acad. Sci. U.S.A.* 24, 279 (1938).

¹⁵⁸ F. C. Schmelkes, *Science* 90, 113 (1934); F. C. Schmelkes and R. K. Jonner, *J. Am. Chem. Soc.* 61, 2562 (1939); F. H. Ungelen and A. Dornow, *Ber.* 73, 44 (1940).

¹⁵⁹ A. Dornow, *Ber.* 73, 156 (1940).

It may be noted that substances of the cyclo pentano perhydro phenanthrene type (for example male and female sex hormones vitamin D) have been reported¹⁵⁹ as delaying the vitamin B₁ avitaminosis (polyneuritic symptoms in pigeons) These results however need confirmation

12 Determination

(a) Chemical Methods

A number of color reactions have been proposed for the determination of vitamin B₁. Reliable quantitative results can be obtained with the first three procedures when carefully controlled conditions are observed

1 **Formaldehyde-azo-test of Kinnersley and Peters**¹⁶⁰ Diazo benzene sulfuric acid in carbonate containing sodium hydroxide solution gives with vitamin B₁ and formaldehyde a red color. Since this reaction attacks the 4 amino group of the pyrimidine nucleus, it is obvious that a deaminated vitamin or thiochrome does not give this reaction. Therefore this reaction is quite valuable for the determination of the natural vitamin. Synthetic compounds of similar structure give the same reaction. Substituents in the thiazolium nucleus influence the reaction.¹⁶¹ This method of vitamin B₁ determination is applicable to rather highly purified solutions only. The presence of reducing substances interferes with the color development. The use of acetone in place of vitamin B₁ also gives the same color with the reagents of this test method.¹⁶²

2 **Thiochrome Test by Jansen** Vitamin B₁ in aqueous solution is oxidized by means of potassium ferricyanate to thiochrome, the fluorescence of which is determined photoelectrically after extraction of the thiochrome with isobutanol.^{163 164 165 166 167} The oxidation is carried out at pH 10. The intensity of the fluorescence is a function of the alkalinity of the solution and of the amount of thiochrome present. Excess potassium ferricyanide destroys the formed thiochrome rapidly. Irradiation also destroys the thiochrome.

¹⁵⁹ J. Sanchez Rodriguez and J. M. S. rda *Z. Vitaminsforsch.* 6, 193 (1937)

¹⁶⁰ H. W. Kinnersley and R. A. Peters *Biochem. J.* 28, 667 (1934); *Ibid.* 32, 1016 (1938)

¹⁶¹ F. Bergel and A. R. Todd *J. Chem. Soc.* 1937, 1504

¹⁶² C. R. Addinall *The Story of Vitamin B₁* Merck & Co. 1937, p. 18

¹⁶³ G. Barger, F. Bergel and A. R. Todd *Nature* 136, 250 (1935); *Ber.* 68, 2, 57 (1935)

¹⁶⁴ B. C. P. Jansen *Rec. trav. chim.* 55, 1046 (1936); H. G. K. Westenbrink and J. Goudsmit *Ibid.* 56, 803 (1937); P. Karrer and U. Kubli *Helv. Chim. Acta* 20, 369 (1937); J. Wendenbauer, O. Huba and C. Becker *Z. ges. exp. Med.* 101, 178 (1937); J. Goudsmit and H. G. K. Westenbrink *Nature* 139, 1108 (1937); K. Ritsert *Deut. med. Wochschr.* 64, 481 (1938); H. Otto and F. Rühmke *Arch. Wochschr.* 17, 1246 (1938)

¹⁶⁵ H. G. K. Westenbrink and J. Goudsmit *Nature* 142, 100 (1938)

¹⁶⁶ C. M. Hills *Biochem. J.* 33, 1906 (1939)

¹⁶⁷ R. G. Booth *J. Soc. Chem. Ind.* 59, 181 (1940)

The results obtained with this method are in fair agreement with those obtained by the biological bradycardia method. It must however be noted that only the free vitamin B₁ is determined by this method, since only the free thiochrome can be extracted with organic solvents. This difficulty can be overcome by digesting the sample with pepsin followed by digestion with taka diastase.¹⁶⁸ Ambiguity arises sometimes through colored fluorescence of other compounds which obscures the effect of thiochrome.¹⁶⁹

3 Colorimetric Method by Prebluda and McCollum¹⁷⁰ Diazotized *p*-amino acetanilide, *p*-amino acetophenone or methyl *p*-amino acetophenone gives with vitamin B₁ red dyes, specific for this vitamin, which can be extracted with organic solvents such as xylene, acetone, isobutanol, etc. The sensitivity of this reaction is increased by the presence of phenol or ethyl alcohol or preferably, both.¹⁷¹ Willstaedt¹⁷² suggests the use of 2,4-dichloro benzene diazonium chloride. Extraction of the formed yellow red dye with ether and separation from by-products by adsorption on calcium hydroxide. This method has been modified by running the analysis with diazotized sulfanilic acid with and without the presence of potassium ferricyanide. Since vitamin B₁ does not give a color under these conditions but other amino compounds which may accompany the vitamin do give colors, the difference between the color values gives a measure of the amount of vitamin B₁ present.¹⁷³

The results of these methods compare favorably with the bioassay values. Vitamin C interferes with the development of the color¹⁷⁴ unless it is first oxidized, for example, by titrating with iodine or by the addition of calcium ions.¹⁷⁵ The phosphorylated forms of vitamin B₁ give the color but the dye formed cannot be extracted by organic solvents such as xylene.¹⁷⁶

4 Gravimetric Method by Nauman¹⁷⁷ This method is based on the production of an orange red precipitate of vitamin B₁ with bismuth potassium iodide.

¹⁶⁸ M. Pyke *J. Soc. Chem. Ind.* 53, Trans. 338 (1939).

¹⁶⁹ M. Pyke *J. Soc. Chem. Ind.* 58, 1021 (1939).

¹⁷⁰ H. J. Prebluda and E. V. McCollum *Science* 84, 488 (1930); *J. Biol. Chem.* 127, 495 (1939). D. Melnick and H. Field *J. Biol. Chem.* 127, 505, 515 (1939).

¹⁷¹ D. Melnick and H. Field *J. Biol. Chem.* 127, 515 (1939).

¹⁷² H. Willstaedt *Nat.wissenschaften* 25, 687 (1937).

¹⁷³ P. Meunier and C. P. Blancpain *Compt. rend.* 208, 768 (1939).

¹⁷⁴ A. D. Emmett, G. Peacock and R. A. Brown *J. Biol. Chem.* 135, 131 (1940).

¹⁷⁵ M. E. Auerbach *J. Am. Pharm. Assoc.* 29, 313 (1940).

¹⁷⁶ D. Melnick and H. Field *J. Biol. Chem.* 127, 531 (1939).

¹⁷⁷ B. Nauman *Science* 85, 290 (1933).

5 Method of Spruyt¹⁷⁸ Vitamin B₁ is separated as the phosphotungstate and reduced with nascent hydrogen. The resulting brown color is believed to be proportional to the amount of vitamin B₁ present.

6 Raybin Reaction¹⁷⁹ Vitamin B₁ in a borax solution of pH 9.6 forms with 2,6 dibromo quinone chloroimide an orange color which gradually decreases in intensity. The color formed can be extracted with chloroform and measured in a photometer.

7 Tauber Reaction¹⁸⁰ Vitamin B₁ and *p*-dimethyl amino benzaldehyde in the presence of acetic acid produce upon evaporation of the acid and addition of fresh acid an intense brick red color. This reaction should not be carried out in the presence of proteins or amino acids which interfere.

Vitamin B₁ in the presence of vitamin B₁ pyrophosphate can be determined by either the thiochrome method (thiochrome pyrophosphate cannot be extracted from an alkaline solution with isobutanol)¹⁸¹ or by condensation with diazo compounds according to Prebluda and McCollum. Vitamin B₁ pyrophosphate gives the same color reactions as does vitamin B₁. The color developed cannot, however, be extracted by organic solvents. For the determination of the total vitamin B₁ present, the material might be hydrolyzed enzymatically, followed by the determination of the then free vitamin B₁.^{181, 182}

(b) Biological Methods

More accurate than any of the described chemical methods for the determination of vitamin B₁ are the biological methods. Either birds, especially the pigeon, or rats are used.

The curative pigeon test by Kinnersley, Peters and Reader uses the disappearance of tonic spasms and convulsions.¹⁸³ Also a prophylactic pigeon test has been worked out. Rats are recommended by the U. S. Pharmacopoeia as test animals in the curative method.^{184, 185} Vitamin B₁ deficiency in rats causes convulsions and paralysis of the lower extremities.

¹⁷⁸ J. P. Spruyt *Chem. Weekblad* 27: 298 (1930). H. W. Acton, S. Ghosh and A. Dutt, *Ind. J. Med. Research* 1933: 103.

¹⁷⁹ H. W. Raybin *Science* 88: 35 (1938).

¹⁸⁰ H. Tauber *Ibid.* 86: 594 (1937).

¹⁸¹ H. G. K. Westenbrink and B. C. P. Jansen *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.* 5: 119 (1938). H. W. Kinnersley and R. A. Peters *Biochem. J.* 28: 667 (1934). 29: 2369 (1935). 32: 1, 16 (1938).

¹⁸² J. Houston and S. K. Lion *Nature* 143: 558 (1939).

¹⁸³ H. W. Kinnersley, R. A. Peters and V. Reader *Biochem. J.* 19: 820 (1928).

¹⁸⁴ F. Hofmeister *Biochem. Z.* 129: 477 (1922). M. I. Smith *U. S. Pub. Health Service Pub. Health Repts.* 24: 116 (1930). E. F. Cook *J. Am. Pharm. Assoc.* 28: 267 (1939).

¹⁸⁵ C. L. Kline, C. D. Tolle and E. M. Nelson *J. Assoc. Official Agr. Chem.* 21: 305 (1938).

which are cured by the administration of the vitamin. The rat growth test involves the determination of the minimum amount of the vitamin required for growth and maintenance¹⁸⁶. The electrocardiographic bradycardia test according to Birch and Harris is based on the decline of the heart rate of rats during avitaminosis¹⁸⁷ and its cure by the administration of the vitamin. Vitamin B₁ may also be determined with chicks by a method¹⁸⁸ in which the degree of postponement of fatal polyneuritic symptoms afforded by the test material is compared with that afforded by various levels of synthetic thiamin chloride hydrochloride. Another method using chicks as assay animals determines the rate of growth produced by vitamin B₁¹⁸⁹.

(c) Biochemical Methods

1. The "Catatorulin"^{189a} test¹⁹⁰ which is often used measures *in vitro* the uptake of oxygen by brain tissue from avitaminotic pigeons. Addition of vitamin B₁ increases proportionately the amount of oxygen consumed.

2. Yeast Fermentation Test. The stimulating effect of vitamin B₁ on alcoholic fermentation has been developed into a quantitative test^{191, 192}. As little as 1 γ may be detected ($\frac{1}{3}$ International Unit).

3. Mold Growth Method. Within certain limits the growth of the mold *Phycomyces blakesleanus* is proportional to the concentration of vitamin B₁ present in a synthetic nutritive medium and has been used for the determination of vitamin B₁ in blood.¹ 0.01 γ can be detected according to this procedure¹⁹³. A modification of this method¹⁹⁴ measures the increase of carbon dioxide formed by the addition of vitamin B₁ to growing *Phycomyces* (sensitivity 0.1 γ).

4. Cocarboxylase can be determined quantitatively by the amount of CO₂ produced by decarboxylation of pyruvic acid by yeast which has

¹⁸⁶ H. C. Sherman and A. Spohn, *J. Am. Chem. Soc.* 45, 2719 (1923). I. P. Chase, *Dissertation*, Columbia, 1928. H. C. Sherman and S. L. Smith, *The Vitamins*, Chem. Al. Catalog Co., 1931.

¹⁸⁷ T. W. Birch and I. J. Harris, *Biochem. J.* 28, 602 (1934).

¹⁸⁸ T. H. Jukes and H. Hettman, *J. Nutrition* 19, 21 (1940).

¹⁸⁹ A. Arnold and C. A. Elvebjem, *Ibid.* 15, 403 (1938).

^{189a} Torulin = vitamin B₁.

¹⁹⁰ R. Passmore, R. A. Peters, and H. M. Sinclair, *Biochem. J.* 27, 842 (1933). H. W. Kinnersley, J. R. O'Brien, and R. A. Peters, *Ibid.* 29, 701 (1935). R. A. Peters, *Ibid.* 32, 7031 (1938).

¹⁹¹ A. S. Schultz, L. Atkin, and C. N. Frey, *J. Am. Chem. Soc.* 59, 949, 2457 (1937). 60, 1514 (1938).

¹⁹² A. S. Schultz, L. Atkin, and C. N. Frey, *Ibid.* 60, 490 (1938).

¹⁹³ W. H. Schopfer, *Z. Vitaminforsch.* 4, 67, 187 (1935). W. H. Schopfer and A. Jung, *Compt. rend. soc. Biol.* 122, 749 (1936). A. P. Meiklejohn, *Biochem. J.* 31, 1441 (1937). H. M. Sinclair, *Ibid.* 32, 2185 (1938).

¹⁹⁴ E. Heyn, *Z. physiol. Chem.* 258, 219 (1939).

been freed from cocarboxylase in the presence of maximum amounts of vitamin B₁ ¹⁹⁵

13 Standards

One International Unit vitamin B₁ = 3 γ of vitamin B₁ hydrochloride, colorless monoclinic plates m p 246-247° (decomp) = 1 U S Pharmacopoeia Unit

This unit replaces the earlier one of 1934 according to which 10 mg of a special adsorbate of vitamin B₁ on fuller's earth represented 1 International Unit. The new Unit is the biological equivalent of the old Unit. The adsorption product which represented the old standard was prepared as follows: 100 kg rice polishings are extracted with water, sufficient sulfuric acid being added to obtain a pH of 4.5. Salicylic acid (0.2%) and toluene are added to prevent bactericidal decomposition. After two days extraction the solution is filtered and shaken for 24 hours with especially adsorptive fuller's earth. After filtration the fuller's earth is dried.

In order to standardize vitamin B₁ preparations of unknown activity, the U S Pharmacopoeia recommends the rat curative method (see above) ¹⁹⁶

1 I U vitamin B₁ corresponds about to ¹⁹⁷ 0.5 Smith Curative Unit ¹⁹⁸ 2.0 Chase Sherman Units ¹⁹⁹ 1.0 Roscoe Unit ²⁰⁰ and 20.0 Milligram Equivalents (Cowgill) ²⁰¹. Units other than I U should no longer be used. Actually it is impossible to translate one unit system into another by factors or by mathematical computation. The above figures are given only as a guide to indicate the approximate order of magnitude for the purpose of facilitating the reading of some of the original literature.

14 Physiology of Plants and Microorganisms²⁰²

Thiamin is a vitamin with respect to animal nutrition. Its occurrence in plants and microorganisms has raised the question as to the physiological significance of this compound in plants and microorganisms. This question can partly be answered. Vitamin B₁ is a true growth promoting substance for such organisms and is needed in small amounts for normal development.

Plants and microorganisms can be classified into two groups, namely those which need an external supply of the growth promoting substance.

¹⁹⁵ S. Ochoa and R. A. Peters *Biochem J.* 32: 1501 (1938).

¹⁹⁶ F. F. Cook *J. Am. Pharm. Assoc.* 28: 267 (1939).

¹⁹⁷ C. R. Addinall *The Story of Vitamin B₁*, Merck Co. 1937.

¹⁹⁸ M. I. Smith *U. S. Pub. Health Service Pub. Health Repts.* 24: 111 (1930).

¹⁹⁹ H. C. Sherman and A. Spohn *J. Am. Chem. Soc.* 45: 2719 (1923). F. F. Chase *Distribution* Columbia 1928. H. C. Sherman and S. I. Smith *The Vitamins*, Chemical Catalog Co. 1931.

²⁰⁰ H. Chick and M. H. Roscoe *Biochem J.* 23: 408 (1929).

²⁰¹ G. R. Cowgill *The Vitamin B Requirement of Man*, Yale University Press 1934.

²⁰² W. J. Robbins *Science* 89: 303 (1939).

and those which are able to synthesize it. Among the lower plants and microorganisms which have the power of synthesizing vitamin B₁ are yeasts, bacteria, fungi, etc. Special examples are *Aspergillus niger*, *Agaricus campestris*, *Absidia glauca*, *Bacterium coli*, *Bacillus pyocyaneus*, *Chlamydomonas*, *Chlorogonium* and *Polytoma obtusum* (see also page 101). Since many of these organisms contain no chlorophyll, it must be concluded that chlorophyll is not an essential factor for the synthesis of the vitamin. It seems significant, however, that plants (peas) raised in the dark contain very little thiamin, whereas the content of this compound in plants increases rapidly in light.⁹² All higher plants are able to synthesize thiamin. It has been demonstrated, for example, for tomatoes, that the vitamin is synthesized in the shoots. In leaves the concentration of vitamin B₁ amounts to a constant value of about 25 International Units per 100 g, regardless of the botanical family.²⁹⁴ Roots do not have the power of synthesizing vitamin B₁, however, they need thiamin for continued cell division in the embryonic region. Vitamin B₁ is essential for the growth of all species of roots investigated.⁹³ Thus, thiamin appears to be a true plant hormone. On the other hand, tomato roots are able to synthesize the pyrimidine portion of thiamin, but require an external supply of the thiazole part. (Experiments done with excised tomato roots.) Pea roots require an external supply of both parts.²⁹⁵ Vitamin B₁ is stored in seeds and more specifically in the outer integuments.

Some plant species (for example, cosmos, camellia, etc.) are said to grow more luxuriantly when the roots obtain, besides the self-synthesized vitamin B₁, some of this vitamin from an outside source. Under very carefully conducted experiments it has not, however, been possible to detect any beneficial effects from added vitamin B₁ when plants were raised from their seeds.⁹⁷ It has been shown that in cuttings and after transplanting vitamin B₁ promotes root growth.²⁹⁸

Higher plants not only synthesize vitamin B₁ but excrete it through the roots. Therefore the soil in the immediate vicinity of plant roots supports a much higher microbial population than that existing outside the plant's

⁹² J. Bonner and J. Greene *Botan. Gaz.* 100, 6 (1938).

⁹³ M. Pyke *Biochem. J.* 34, 330 (1940).

⁹⁴ J. Bonner *Am. Chem. Soc. Div. Agr. Food Chem. Meeting*, Sept. 1939, Abst. 13. F. W. Went, J. Bonner and G. C. Warner *Science* 87, 170 (1938).

⁹⁵ P. Kögl and A. J. Haagen-Smit *Z. physiol. Chem.* 243, 209 (1936). J. Bonner *Science* 85, 183 (1937). W. J. Robbins and M. A. Bartley *ibid.* 85, 246 (1937). W. J. Robbins, M. A. Bartley, A. C. Han and L. R. Richardson *Proc. Natl. Acad. Sci. U. S.* 23, 384 (1933).

⁹⁶ D. I. Arnon *Science* 92, 264 (1940).

⁹⁷ J. Bonner *Am. Chem. Soc. Div. Agr. Food Chem. Meeting*, Sept. 1939, Abst. 13. F. W. Went, J. Bonner and G. C. Warner *Science* 87, 170 (1938).

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²⁰⁰ H. Chick and M. H. Roscoe *Biochem J.* 23: 498 (1929)

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²⁰³ J. Bonner and J. Greene *Botany* 100: 226 (1938).

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²⁰⁵ J. Bonner *Am. Chem. Soc. Div. Agr. Food Chem. Meeting* Sept. 1939, Abst. 13. F. W. Went, J. Bonner and G. C. Warner *Science* 87: 170 (1938).

²⁰⁶ F. Kögl and A. J. Haagen-Smit *Z. physiol. Chem.* 243: 209 (1936). J. Bonner *Science* 85: 183 (1937). W. J. Robbins and M. A. Bartley *Ibid.* 85: 246 (1937). W. J. Robbins, M. A. Bartley, A. C. Hagan and L. R. Richardson *Proc. Natl. Acad. Sci. U. S. A.* 23: 388 (1937).

²⁰⁷ D. I. Arnon *Science* 92: 64 (1940).

²⁰⁸ J. Bonner *Am. Chem. Soc. Div. Agr. Food Chem. Meeting* Sept. 1939, Abst. 13. F. W. Went, J. Bonner and G. L. Warner *Science* 87: 10 (1938).

zone of influence²⁰⁹ A great number of bacteria yeasts and fungi²¹⁰ are known to require an external supply of thiamin for example, *Staphylococcus aureus*,²¹¹ *Phycomyces blakesleanus*²¹² and *Polytomella caeca*²¹³ Most of them are parasites, some are saprophytes Some organisms which need an external supply of thiamin are able to synthesize it from the thiazole and the pyrimidine intermediate, for example, *Phycomyces blakesleanus* *Phytophthoras*, on the other hand, is unable to do so and resembles a higher animal in this respect

A few organisms are known to be able to synthesize only one part of the thiamin molecule either the pyrimidine part, for example *Polytoma caudatum* and *Chilomonas paramecium*²¹⁴ or the thiazole part These organisms however can combine the portion synthesized with the portion obtained from outside when properly administered

The mechanism of the action of thiamin in plants is not known It is however reasonable to assume that thiamin functions similarly in plants as it does in animals (see page 135)

It should finally be noted that vitamin B₁ also promotes alcoholic fermentation by yeast²¹⁵ which effect can be used for the determination of the vitamin (see page 131) The growth of acetic acid bacteria is also stimulated by the vitamin²¹⁶

15 Animal Physiology

(a) Metabolism of Vitamin B₁

Vitamin B₁ is completely absorbed in the small gut and partly secreted in the gastric juice probably by means of diffusion²¹⁷ It has already been mentioned that vitamin B₁ is not stored in the organism, but relatively higher concentrations are found in the liver, kidneys, heart muscles and brain²¹⁸ The amount actually present is enough to maintain proper life only for a few days A daily intake of vitamin B₁ is, therefore, necessary

²⁰⁹ P M West *Nature* 144 10-0 (1939)

²¹⁰ R J Williams and R R Roehm *J Biol Chem* 87 581 (1930) H Burgeff *Ber deut biol Ges* 52 384 (1934) W H Schopfer *Arch Mikrobiol* 6 510 (1935) F Kögl and N Fries *Z physiol Chem* 249 93 (1937)

²¹¹ B C J G Knight *Nature* 139 628 (1937) *J Soc Chem Ind* 56 445 (1937) *Biochem J* 31 731 (1937)

²¹² W H Schopfer *Compt rend* 200 1965 (1935) *Z Vitaminforsch* 4 187 (1937) W H Schopfer and A Jung *Compt rend* 204 1500 (1937) H M Sinclair *Nature* 140 361 (1937)

²¹³ A Lwoff and H Duss *Compt rend* 205 630 (1937)

²¹⁴ A Lwoff and H Duss *Ibid* 205 756 (1937)

²¹⁵ A Schultz L Atkin and C N Frey *J Am Chem Soc* 59 948 (1937)

²¹⁶ T J Paley *Mikrobiol* 7 843 (1938)

²¹⁷ M Stockholm T L Althausen and H J Borson *Proc Soc Exptl Biol Med* 46 387 (1941)

²¹⁸ H G K. Westenbrink *Arch nterland physiol* 17 560 (1932) 19 116 (1937)

The organism absorbs only as much vitamin B₁ as is needed for the time being. All excess is excreted and to a small extent destroyed. Even intramuscular injection causes immediate excretion in the urine. A vitamin B₁ deficiency can be detected from the amount of vitamin excreted in the urine: a normal person should excrete 20–80 International Units per day. Vitamin B₁ is also secreted in milk and eggs.

Vitamin B₁ is present in the organism both in the free and esterified forms. The pyrophosphoric acid ester has been identified as cocarboxylase. The presence of vitamin B₁ monophosphate in the animal organism is suspected. Both vitamin B₁ and cocarboxylase occur also in combination with proteins.²¹⁹

The vitamin B₁ may be absorbed in the intestines in the free or in the phosphorylated form. Phosphatase from the duodenum (pigs) can phosphorylate vitamin B₁ *in vitro*.²²⁰ But it seems that vitamin B₁ circulates in blood plasma and in cerebrospinal fluid²²¹ in the free form (concentration about 1 γ per 100 cc) which diffuses readily and passes into tissue fluid, cerebrospinal fluid, urine and cells of the body. A constant phosphorylation and dephosphorylation take place inside the cells. Liver, kidney²²² and to a lesser extent muscle and brain²²³ can convert the vitamin into cocarboxylase. Nucleated blood cells and probably all nucleated animal cells can phosphorylate vitamin B₁.²²⁴ The theory has been advanced that the mammalian non-nucleated erythrocytes obtain their cocarboxylase while in the nucleated form within the bone marrow.²²⁴

In blood the cocarboxylase is entirely confined to the blood cells, whereas the free vitamin occurs only in the serum. Of the total vitamin B₁ in blood nearly 90% is in the phosphorylated form. There seems to be a constant level of about 0.5 γ of free vitamin B₁ per 100 cc of blood.

(b) *Physiological Action of Vitamin B₁*

Vitamin B₁ in the form of its pyrophosphoric acid ester is intimately concerned with the carbohydrate metabolism. Specifically this vitamin is involved in the utilization of pyruvic acid,²²⁵ an intermediary degradation product of carbohydrates both in alcoholic fermentation and in tissue me-

²¹⁹ R. S. Goodhart and H. M. Sinclair, *Biochem. J.* **33**, 1099 (1939).

²²⁰ H. Tauber, *Science* **86**, 180 (1937); *Enzymologia* **2**, 171 (1937); *J. Biol. Chem.* **123**, 499 (1938).

²²¹ H. M. Sinclair, *Biochem. J.* **33**, 1816 (1939).

²²² H. O. K. Westermark and J. Goudsmit, *Enzymologia* **5**, 307 (1938).

²²³ S. Ochoa and R. A. Peters, *Biochem. J.* **32**, 1401 (1938).

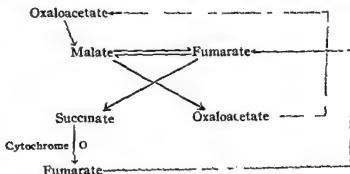
²²⁴ R. L. Goodhart and H. M. Sinclair, *Id.* **33**, 1099 (1939).

²²⁵ See the review by R. A. Peters, *Current Science* **5**, 207 (1936).

can be obtained by the addition of oxaloacetic acid or of pyruvic acid in the presence of carbon dioxide. The rate of pyruvate utilization in these experiments depended upon the concentration of carbon dioxide used, thus indicating the formation of oxaloacetic acid according to reaction (2). This pyruvic acid metabolism through the citric acid cycle occurs apparently in many animal tissues, such as liver, brain and several others, in plants, molds and in those types of bacteria²⁴⁰ in which citrate, succinate, malate or oxaloacetate²⁴¹ is formed, but seems to be of minor importance in skeletal²² and heart muscles.²⁴³

The concept that vitamin B₁ is concerned in reaction (2) explains a number of other observations. Thus, in contrast to the apparent universal decarboxylation of α oxo carboxylic acids by yeast, animal tissues have been found to utilize pyruvic acid exclusively.²⁴⁴ In *in vitro* experiments with rat kidney slices the synthesis of citric acid from pyruvic acid was found to be markedly accelerated by vitamin B₁.²⁴⁵ *In vivo* a deficiency of vitamin B₁ in the diet of rats results in decreased excretion of citric acid through the urine,²⁴⁶ but on the basis of the results obtained from paired feeding experiments it is indicated that the decrease in citric acid secretion is correlated with a diminished intake of food rather than with absence of vitamin B₁ *per se*.²⁴⁷

✓ (b) *The Succinic Acid Cycle* The succinic acid cycle²⁴⁸ may be considered as a variation of the citric acid cycle in that citric acid is not in



involved and in that there is a difference in the nature of the oxidative and reductive reactions. The common principle of both cycles is the reduction

²⁴⁰ H. G. Wood and C. H. Werkman *Biochem J.* 34, 129 (1940).

²⁴¹ A. I. Virtanen and T. Laine *Nature* 141, 748 (1938).

²⁴² H. A. Krebs and L. V. Eggleston *Biochem J.* 34, 442 (1940); H. A. Krebs *Ibid.* 34, 460 (1940).

²⁴³ H. A. Krebs and L. V. Eggleston *Ibid.* 34, 1383 (1940).

²⁴⁴ G. K. McGowan and R. A. Peters *Ibid.* 31, 1637 (1937).

²⁴⁵ E. S. G. Barron and C. M. Lyman *Science* 92, 337 (1940).

²⁴⁶ H. A. Sober, M. A. Lipton and C. A. Elvehjem *J. Biol. Chem.* 134, 605 (1940).

²⁴⁷ A. H. Smith and C. E. Meyer *Ibid.* 139, 227 (1941).

²⁴⁸ K. Laki, F. B. Straub and A. Szent Györgyi *Z. physiol. Chem.* 247, 1 (1937).

of one part of the oxaloacetate (or pyruvate) at the expense of the oxidation of another part ²⁴⁹

According to the succinic acid cycle oxaloacetate is reduced to malate at the expense of an oxidation of pyruvate. Thus this reaction may be formulated ⁴⁹



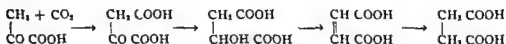
The malate formed according to (3) may react further in two different ways. It may react with another molecule of pyruvate (4)



Or malate may according to the succinic acid cycle be converted into fumarate and finally into succinate.

It is postulated that all these reactions occur at considerably different rates in various tissues. They serve well to explain the experimental findings. The succinic acid cycle appears to be involved in the metabolism of pyruvic acid through reaction (2) in various bacteria and to a certain extent also in animal tissues.

In certain bacteria anaerobic fermentation of pyruvate yields ⁴⁹ malate, fumarate and succinate and results in an accumulation of the latter as postulated by the succinic cycle concept. This reaction can then be written ²⁵⁰



and occurs in *Propionibacterium B. coli* and other species. In the case of *B. coli* it was shown ²⁵¹ that the yield of succinate depends upon the pressure of CO₂. Succinic acid formation from pyruvic acid has also been demonstrated in certain animal tissues such as for example in the kidney of rats ²⁵. In experiments in which the fixation of radioactive carbon dioxide by bacteria was studied the radioactive element was actually found in the succinic acid isolated from the reaction products ²⁵² and by degradation of the succinic acid it was shown that the radioactive carbon is exclusively in the carboxyl groups of the acid ²⁵⁴

²⁴⁹ H. A. Krebs and L. V. Eggleston *Biochem. J.* 34, 1343 (1940)

²⁵⁰ H. G. Wood and C. H. Werkman *Ibid.* 34, 7 (1940)

²⁵¹ S. R. Eidsen *Ibid.* 32, 187 (1938)

²⁵² F. S. G. Barron, C. M. Lyman, M. A. Lipton and J. Goldfinger *Proc. Am. Soc. Biol. Chem.* 1941 XI

²⁵³ H. G. Wood, C. H. Werkman, A. Hemingway and A. O. N. *J. Biol. Chem.* 139, 265 (1941)

²⁵⁴ H. G. Wood, C. H. Werkman, A. Hemingway and A. O. N. *Ibid.* 139, 277 (1941)

The reaction mechanism in certain other bacteria such as in *Staphylococcus*²⁵⁵ and *Lactobacillus delbrueckii* is somewhat different^{256 257} In these organisms the reaction proceeds mainly through reaction (3) and results in an accumulation of acetic acid The other reaction products are totally metabolized

In animal tissues for example in the brain and in certain bacteria, lactic acid is accumulated,^{255 258 259} which originated from the succinic cycle according to reaction (4)²⁴⁹

In most animal tissues and in bacteria, all these reactions occur at the same time but at different rates Thus, in quantitative experiments with brain tissue,²⁶⁰ pyruvic acid was utilized in the following manner 67% was completely metabolized according to the citric acid cycle yielding only CO₂, 29.5% was carried through the succinic acid cycle yielding besides CO₂, acetic acid and lactic acid

Certain other explanations for the reaction mechanism of the pyruvic acid metabolism in the presence of vitamin B₁ have been postulated at various times They all differ from the mechanism as presented in the previous paragraphs in that reaction (2) the initial formation of oxaloacetic acid is not postulated As the result an involvement of the citric and succinic acid cycles was not considered Thus the formation of acetic acid has been explained as decarboxylation (according to reaction (1)) with simultaneous dehydrogenation²⁶¹



or



The occurrence of lactic acid has been explained as a dismutation^{262 263 264}



It has been suggested that the dehydrogenations and dismutations are accomplished through some flavin adenine enzyme systems²⁶⁵ while only the decarboxylations are carried out by the vitamin B₁ pyrophosphate enzyme system These explanations do not appear attractive in the light of the previously discussed hypothesis and the evidence for an intermediary formation of oxaloacetic acid by means of the vitamin B₁ enzyme system

²⁵⁵ E S G Barron and C M Lyman *J Biol Chem* 127 143 (1939)

²⁵⁶ F Lipmann *Enzymologia* 4 64 (1937) *Nature* 140 75 (1937) 143 436 (1939)

²⁵⁷ G M Hills *Biochem J* 32 383 (1938)

²⁵⁸ H A Krebs *Nature* 138 288 (1936)

²⁵⁹ H A Krebs *Biochem J* 31 661 (1937)

²⁶⁰ C Long *Ibid* 32 1711 (1938)

²⁶¹ F Lipmann *Enzymologia* 4 64 (1937) *Nature* 140 25 (1937) 143 436 (1939)

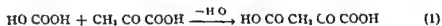
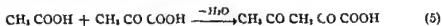
²⁶² H A Krebs *Nature* 138 288 (1936)

²⁶³ E S G Barron and C M Lyman *J Biol Chem* 127 143 (1939)

²⁶⁴ H A Krebs *Biochem J* 31 661 (1937)

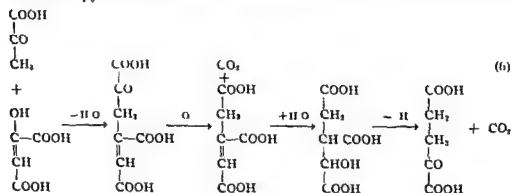
²⁶⁵ F Lipmann *Nature* 143 281 (1939)

The initial formation of oxaloacetate from pyruvate under the influence of diphospho vitamin B₁ also makes a condensation of pyruvic acid with other acids plausible. Thus it might be possible⁶ that acetic acid condenses with pyruvic acid (reaction (5)) in strict analogy to the condensation of CO with pyruvic acid (reaction (1))



Actually the formation of acetoacetate in pigeon⁶⁶ and chicken⁶⁷ liver has been demonstrated to be a function of vitamin B₁, pyrophosphate and might be explained by an initial formation of acetylpyruvate according to reaction (5)

The citric and the succinic acid cycles are probably not the only reactions involved in the utilization of pyruvic and oxaloacetic acids. At least one other mechanism must be assumed, since in radioactive α keto glutaric acid obtained in rat liver from pyruvic acid and radioactive sodium bicarbonate the radioactive carbon atom is fixed solely in the carboxyl group which is in α position to the keto group as evident from degradation reactions²⁰⁴. Thus this α keto glutaric acid cannot be formed according to the citric acid cycle which would require an equal distribution of the radioactive carbon in both carboxyl groups. Another series of reactions (6) has therefore been postulated⁶⁸ for the formation of this acid from oxaloacetic and pyruvic acids



So far the action of vitamin B₁ in the carbohydrate metabolism has been discussed only in its relation to the utilization of pyruvic acid. While this is apparently the most important action other observations may be men-

⁶⁶ H. A. Krebs and L. V. Eggleton *Biochem J.* 34, 1383 (1940)

⁶⁷ I. S. G. Barron, C. M. Lym, M. A. Lipton and J. Goldinger *J. of Am. Soc. Biol. Chem.* 194, 41

⁶⁸ H. C. Wood, C. H. W. Krieger, A. Hemingway and A. O. Nier *J. Biol. Chem.* 139, 483 (1941)

tioned concerning a disturbed carbohydrate metabolism during vitamin B₁ deficiency. Thus, glycogen, the specific animal carbohydrate, requires the presence of this vitamin to be properly metabolized. An increase of the glycogen content in liver and in heart muscles has been observed in pigeons during avitaminosis.⁶⁹ In the urine of children suffering from vitamin B₁ deficiency, methyl glyoxal is found.²⁷⁰ In rabbits intramuscular injection of vitamin B₁ causes hypoglycemia in amounts of 0.75 mg, while 2 mg or more bring about marked hyperglycemia.²⁷¹ In man it has generally been observed that an increased carbohydrate metabolism, for example, after an intake of large amounts of carbohydrates during physical labor or during fever periods, requires an increased vitamin B₁ utilization.

The fat metabolism is influenced by vitamin B₁ only in so far as the synthesis of fat from carbohydrates is concerned that is, the metabolism of ingested fats apparently does not need any vitamin B₁. On the other hand the utilization of acetic acid as an intermediate in the carbohydrate breakdown appears to be accelerated in bacteria by the presence of vitamin B₁.²⁷ Fat synthesis in experimental animals for example in pigeons and in rats, on a pure carbohydrate diet, is dependent upon the presence of vitamin B₁.^{272, 274}

The water metabolism in the organism also seems connected with the action of vitamin B₁ probably through the carbohydrate metabolism. Edema and water imbibitions in the heart and in other organs are symptoms of B₁ avitaminosis. Vitamin B₁ also takes part in the regulation of the nervous system. During nerve excitement, two compounds are liberated from the nerve: acetylcholine and vitamin B₁.²⁷⁵ It has been shown experimentally that vitamin B₁ cannot bring about contractions of the gut, whereas the acetylated vitamin B₁ has the power to do so,²⁷⁶ as does acetylcholine. Acetyl thiamin like acetylcholine can be hydrolyzed enzymatically perhaps even by the same acetylcholine esterase.²⁷⁷ It has, therefore been suggested that actually not vitamin B₁ but an ester is

⁶⁹ E. Abderhalden and W. Wertheimer *Arch. ges. Physiol. (Pflüger's)* 233 395 (1933)

²⁷⁰ A. Geiger and A. Rosenberg *Klin. Wochschr.* 12 1258 (1933)

²⁷¹ C. Ortoleva *Biochim. terap. sper.* 25 511 (1938)

²⁷² J. H. Quastel and D. M. Webley *Nature* 144 633 (1939)

²⁷³ E. W. McHenry *Science* 86 00 (1937) E. W. McHenry and G. Gavin *J. Biol. Chem.* 125 653 (1938) E. W. McHenry and G. Gavin *Ibid.* 128 45 (1939)

²⁷⁴ E. W. McHenry *J. Physiol.* 89 287 (1937) H. E. Longenecker, G. Gavin and E. W. McHenry *J. Biol. Chem.* 134 693 (1940)

²⁷⁵ B. Minz and R. Agid *Compt. rend.* 205 576 (1937) L. Binet and B. Minz *Arch. intern. physiol.* 42 281 (1936) B. Minz *Compt. rend. soc. biol.* 127 1251 (1938)

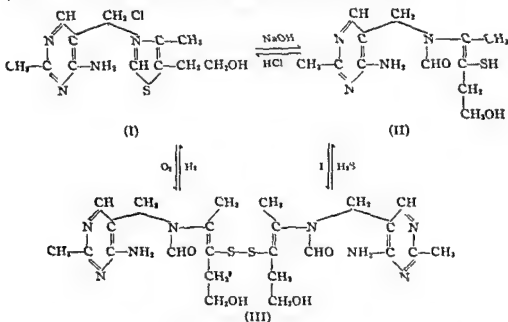
²⁷⁶ R. Kuhn, T. Wieland and H. Huebsehmann *Z. physiol. Chem.* 259 48 (1931)

²⁷⁷ L. Massart and R. Dufaut *Naturwissenschaften* 27 567 (1939) H. Sullmann and H. Berkhäuser *Schweiz. med. Wochschr.* 69 648 (1939) L. Massart and R. Dufaut *Enzymologia* 7 385 (1939)

liberated from the nerves⁷⁵ It should however be noted that one would expect rapid removal of a mediator of nerve impulses from the site of action Thus acetylcholine is made inactive hydrolytically by choline esterase The enzymatic hydrolysis of acetyl thiamin by horse serum and by brain extracts proceeds however very slowly⁷⁹

(c) *Mechanism of the Vitamin B₁ Action*

Vitamin B₁ takes part in tissue oxidations of carbohydrates It might therefore, be expected that vitamin B₁ acts as a compound capable of reversible oxidation and reduction This is apparently the case Vitamin B₁ (I) represents the reduced form and can be oxidized to a disulfide (III)⁸⁰ This oxidation occurs under physiological conditions for ex



ample, at pH 7.5 with hydrogen peroxide or oxygen from air⁸¹ but can also be brought about by oxidation in alkaline solution with iodine. On the other hand the disulfide can be reduced to the thiol form (II) of vitamin B₁ by hydrogen sulfide, glutathione or cysteine⁸¹ and the thiol form is converted into the vitamin by acids such as hydrochloric acid. In

⁷⁵ H. U. Graf and A. v. Muralt *Angew. Chem.* 52, 465 (1939).

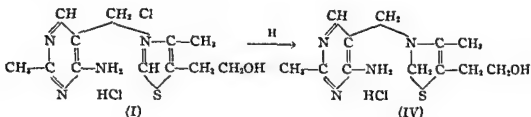
⁷⁹ H. Söllmann and H. Brückhäuser *Schweiz. med. Hochsch.* 69, 648 (1939). L. M. Sarr and R. Dufast *Naturwissenschaften* 27, 667 (1939). D. Glick and W. Antopol *Proc. Soc. Exptl. Biol. Med.* 42, 396 (1939).

⁸⁰ O. Zima and R. R. Williams *Ber.* 73, 941 (1940).

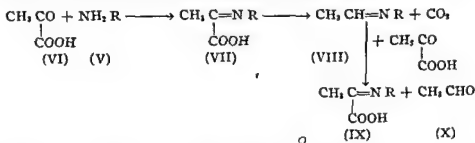
⁸¹ O. Zima, K. Ritsert and T. Moll *Z. physiol. Chem.* 267, 10 (1941).

body fluids and in tissues, the reduction apparently does not stop at the thiol stage. The disulfide shows the full biological activity of the vitamin itself and is about five times less toxic than the vitamin.

The possibility that vitamin B₁ represents not the reduced but the oxidized form has also been investigated. Actually by mild reducing agents for example by the action of hydrosulfite in sodium carbonate solution one atom of hydrogen is absorbed²²³ (I → IV). The reduced form is a reducing agent but is non autooxidizable. It is also devoid of vitamin activity. Furthermore upon pyrophosphorylation the molecule becomes more resistant to the action of reducing agents²²⁴. The possibility therefore that vitamin B₁ acts according to this scheme can be discarded.



It has also been suggested that vitamin B₁ may act through its amino group according to the Langenbeck cycle²²⁵. In this system all primary amines (V) which have been investigated decarboxylate α keto-carboxylic acids (VI) to the corresponding lower aldehyde through the formation of an imino acid (VII) which decomposes into carbon dioxide and an aldimine (VIII). The latter reacts with another molecule of the keto acid thus yielding again one mol of imino acid (IX) and one mol of aldehyde (X). This reaction mechanism is however very improbable since in the actual test system for the Langenbeck cycle vitamin B₁ was found to be completely inactive²²⁶.



(d) Relation of Vitamin B₁ to Other Vitamins, to Hormones, and Minerals

The close relation of vitamin B₁ to the carbohydrate metabolism becomes apparent from the relation of the vitamin to the hormone of the thyroid gland, thyroxine. Thyroxine secretion increases the intensity of the general metabolism and hence necessitates an increased amount of vita-

²²³ F. Lipmann *Nature* **138** 1097 (1937)

²²⁴ B. S. G. Barron and C. M. Lyman *Science* **92** 337 (1940)

²²⁵ *Ergeb. Enzymforsch.* **2** 314 (1933)

²²⁶ K. G. Stern and J. I. Melnick *J. Biol. Chem.* **131** 597 (1939)

min B₁ ^{286 287 288 289 290} A similar relationship can be demonstrated for vitamin B₁ and insulin. Vitamin B₁ increased the sugar tolerance of rats in insulin hypoglycemia experiments ²⁹¹

A close relationship exists between one of the hormones of the adrenal cortex and vitamin B₁. During vitamin B₁ avitaminosis a hypertrophy of the adrenal cortex occurs. Since one of the hormones of the adrenal cortex regulates the lipid content of blood, hypertrophy of the adrenal gland causes increase of the cholesterol content of blood. If during B₁ avitaminosis extracts of the adrenal cortex are injected, hypertrophy of the gland does not occur and the cholesterol content of the blood does not increase. Hypertrophy of the gland, on the other hand, can apparently be cured by vitamin B₁ intake.

It has been reported that substances of the cyclopentano perhydrophenanthrene type, for example, female and male sex hormones and vitamins D ²⁹ delay symptoms of vitamin B₁ deficiency. These results, however, need confirmation.

The relation of vitamin B₁ to the other vitamins also needs further study. Vitamin A is said to act antagonistically, since increase of the vitamin A intake increases the symptoms of vitamin B₁ deficiency. On the other hand, vitamin A deficiency in rats on a diet containing normal amounts of B₁ causes the appearance of symptoms of a B₁ deficiency (pyruvic acid in the blood) which can be cured by increased B₁ intake ²⁹⁴

Interesting is the relationship of vitamin B₁ to the zinc metabolism. During avitaminosis the zinc contents of the blood, ²⁹² the toenails, finger nails and skin are reduced to half their normal values ²⁹³. In natural food stuffs there seems to be a correlation of the amount of zinc and vitamin B₁ ²⁹³

A possible relation of vitamin B₁ to manganese has been observed. When rats were fed 50 International Units per day, there resulted, after one generation, interference with lactation, loss of maternal instinct, cannibalism and progressive loss of fertility. When small amounts of manganese chloride (2 mg per day) were added to the diet, all these effects disap-

²⁸⁶ H. E. Himwich, W. Goldfarb and G. R. Cowgill, *Am. J. Physiol.* 99: 689 (1933).

²⁸⁷ G. R. Cowgill and M. L. Palmieri, *Ibid.* 105: 146 (1933).

²⁸⁸ B. Sure and K. S. Buchanan, *J. Nutrition* 13: 513 (1937).

²⁸⁹ V. A. Drilling and C. R. Sherwood, *Am. J. Physiol.* 124: 683 (1938).

²⁹⁰ R. A. Peters and R. J. Rosviter, *Biochem. J.* 33: 1140 (1939).

²⁹¹ J. C. Burke and A. R. McIntyre, *J. Pharmacol.* 64: 465 (1938).

²⁹² W. G. E. Eggleston, *Chinese J. Physiol.* 15: 33 (1940).

²⁹³ W. G. E. Eggleston, *Biochem. J.* 33: 403 (1939).

²⁹⁴ H. v. Euler and B. Höglberg, *Naturwissenschaften* 27: 769 (1939).

²⁹⁵ J. Sanchez Rodriguez and J. M. Sarda, *Z. Naturforsch.* 6: 193 (1951).

peared²⁹⁶ It has also been noted^{297 298} that Mn^{++} greatly stimulates the carboxylase system if present in appropriate concentrations

16 Avitaminosis and Hypovitaminosis

Severe cases of vitamin B₁ deficiency in man have been and still are very common in the tropics, especially in the Philippines and India and in Japan and are due to poor nourishment, the food consisting mainly of polished rice In America and Europe, severe cases are only occasionally observed and are caused, for example, by chronic alcoholic addiction, by pregnancy, by toxic agents such as nicotine, lead, thallium, arsenic and mercury, etc Cases of more or less severe hypovitaminosis are quite common in the Western countries due to inadequate nutrition

The symptoms generally ascribed to vitamin B₁ deficiency are mostly symptoms of combined deficiencies of several vitamins of the B group, especially B₂, nicotinic amide, B₆ etc Vitamin B₁ deficiency in man affects first of all the emotions and the tonus of the nervous system Further symptoms are loss of appetite (anorexia), unusual susceptibility to fatigue combined with lower physical endurance, gastrointestinal disturbances muscular weakness pains and paraesthesia in arms and legs, edema in ankles and face and decrease of the blood pressure In more severe cases the entire nervous system is affected and symptoms of polyneuritis and neuralgia occur The specific polyneuritis due to vitamin B₁ deficiency is bilateral, symmetrical and involves predominantly the lower extremities²⁹⁹ Muscle cramps of the calf are often noted and, at later stages, calf muscle atrophy The position sense in the toes is disturbed and foot drop is manifested The typical form of beriberi, the severe disease of the tropics, is accompanied by symptoms such as lameness ataxia disturbance of the motor and sensory nerves followed by labored breathing and hypertrophy of the right heart and finally death from heart failure Cardiovascular dysfunction may also arise from mild but continued deficiency of vitamin B₁³⁰⁰

The symptoms of vitamin B₁ deficiency in rats resemble very closely the symptoms of human beings In addition to these, there is a marked influence upon the growth of young animals More severe avitaminosis causes convulsions and paralysis of the lower extremities

²⁹⁶ D Perla *Proc Soc Exptl Biol Med* 37 169 (1937) *Science* 89 132 (1939)

²⁹⁷ K. Lohmann and P Schuster *Biochem Z* 294 183 (1937)

²⁹⁸ S Ochoa and R A Peters *Biochem J* 32 1501 (1938)

²⁹⁹ N Jolliffe *Minnesota Med* 23 542 (1940)

³⁰⁰ S Weiss and R W Wilkins *Tr A Am Physicians* 51 341 (1936) *Ann Internal Med* 11 104 (1937) W A Jones and B Sure *J Lab Clin Med* 22 991 (1937)

The symptoms in pigeons which have been used for the biological detection of the vitamin are quite characteristic. These birds first show lassitude and eat little. They sit with ruffled feathers and draw their heads far back and upside down (head retraction opisthotonus). Convulsions may follow. Two or three days after these symptoms appear the bird dies.

(a) Clinical Test Methods

A diagnosis for vitamin B₁ deficiency can be carried out by a number of different procedures. The amount of this vitamin present in blood and in urine can be determined. The cocarboxylase content in blood can also be used as an assay procedure. Finally a determination of the amount of pyruvic acid present in blood or in urine can serve as an indication for a vitamin B₁ deficiency. In addition to these direct methods determinations of the tissue saturation with vitamin B₁ may be carried out in which the effects of defined doses of vitamin B₁ are measured by any of the indicated methods before and after administration.

Urine Tests The urinary output of vitamin B₁ is closely related to the state of nutrition of the body. Healthy humans on an adequate diet excrete between 50 and 150 γ vitamin B₁ per day^{301 302 303 304}. Lower values are found during vitamin B₁ deficiency³⁰⁵ pregnancy³⁰⁴ and a variety of diseases^{303 306}. Vitamin B₁ pyrophosphate (cocarboxylase) does not appear normally in the urine.

For the actual determination of vitamin B₁ a number of different methods have been recommended. The *bradycardia assay*³⁰⁷ for example has given most satisfactory results. The *thiochrome method* must be modified since urine contains fluorescent compounds which interfere with the thiochrome determination. Various modifications of the basic method have been recommended^{308 309 309 310 311} and have been applied successfully as

³⁰¹ J. Houston, S. K. Kon and S. V. Thompson *J. Soc. Chem. Ind.* 58, 651 (1939). J. Houston and S. K. Kon *Nature* 143, 558 (1939).

³⁰² E. Bassett *Klin. Wochschr.* 17, 1237 (1938).

³⁰³ H. Schroeder *Ibid.* 18, 148 (1939).

³⁰⁴ H. G. K. Westenbrink and J. Goudsmit *Arch. nte land Physiol.* 23, 79 (1938).

³⁰⁵ L. J. Harris, P. C. Leong and C. C. Ungley *Lancet* 234, 539 (1938). L. J. Harris and P. C. Leong *Ibid.* 1, 886 (1936).

³⁰⁶ Y. L. Wang and L. J. Harris *Biochem. J.* 33, 1356 (1939). Y. L. Wang and J. Yudkin *Ibid.* 34, 343 (1940).

³⁰⁷ T. W. Birch and L. J. Harris *Ibid.* 28, 607 (1934).

³⁰⁸ J. Marrack and H. P. Hollering *Lancet* 236, 325 (1939).

³⁰⁹ G. M. Hills *Biochem. J.* 33, 1966 (1939).

³¹⁰ H. G. K. Westenbrink, J. Goudsmit and B. C. P. Jensen *Nature* 139, 1108 (1937). *Rec. trav. chim.* 56, 803 (1937). *Acta Bras. Aca. Ci. d. Physiol. Pharmacol. Microbiol.* 8, 91, 119 (1938).

³¹¹ M. Jowett *J. Soc. Chem. Ind.* 58, 536 (1939). *Biochem. J.* 34, 1343 (1940).

clinical test methods especially when larger quantities of the vitamin are being assayed such as in saturation tests. The *colorimetric method by Prebluda and McCollum* has also been applied successfully to the assay of vitamin B₁ in urine³¹². The *yeast fermentation method* offers promising results for urine tests^{313, 314}. Since urine contains, however, other substances which stimulate the fermentation, it has been recommended³¹⁵ to carry out a determination before and after an oxidative inactivation of the vitamin. In a parallel test in which vitamin B₁ has been added to the urine the efficiency of the inactivation procedure is evaluated.

Pyruvic acid determinations^{316, 317, 318, 319, 320, 321, 322, 323, 324, 325} can be made to detect a state of vitamin B₁ deficiency. One assay procedure is based on the isolation of sodium pyruvic acid 2,4 dinitrobenzoate and the red color developed by the latter with strong alkali. In practice, the urine (or the blood) is mixed with trichloroacetic acid (or in the case of blood, better with tungstic acid) and a solution of 2,4 dinitro phenyl hydrazine in diluted hydrochloric acid solution is added. The hydrazones formed are extracted with ethyl acetate followed by an extraction with a sodium carbonate solution. Finally a sodium hydroxide solution is added and the color developed is measured. This method is of course, not specific for the determination of pyruvic acid since other α keto carboxylic acids such as acetoacetic acid, oxaloacetic acid and α keto glutaric acid, give the same reaction. Pyruvic acid can also be estimated by determination of bisulfite binding substances. According to this procedure urine is mixed with solutions of oxalic acid and of sodium bisulfite. After an appropriate length of time, the excess of bisulfite is removed and the bisulfite bound substances are titrated iodometrically.

The amount of pyruvic acid in the urine of the normal and vitamin B₁ deficient man has not been studied systematically. In rats the amount excreted increases during vitamin B₁ deficiency 200–400% but is also a

³¹² D. Melnick and H. Field *J Biol Chem* 123 83 (1938) 130 97 (1939) *Proc Soc Exptl Biol Med* 38 723 (1938)

³¹³ A. S. Schultz, L. Atkin and C. N. Frey *J Am Chem Soc* 59 948 2457 (1937) 60 1514 (1938)

³¹⁴ A. S. Schultz, L. Atkin and C. N. Frey *Proc Soc Exptl Biol Med* 38 404 (1938)

³¹⁵ A. S. Schultz, L. Atkin and C. N. Frey *J Biol Chem* 136 713 (1940)

³¹⁶ B. S. Platt and G. D. Lu *Quart J Med* 5 355 (1936) G. D. Lu *Biochem J* 33 249 (1939)

³¹⁷ F. P. Clift and R. R. Cook *Biochem J* 26 1788 (1932)

³¹⁸ G. G. Banerj and L. J. Harris *Ibid* 33 1346 (1939)

³¹⁹ M. Shils, H. G. Day and E. V. McCollum *Science* 91 341 (1940)

³²⁰ H. A. Harper and H. J. Denel *J Biol Chem* 137 233 (1941)

³²¹ D. Klein *Ibid* 137 311 (1941)

³²² E. M. Case *Biochem J* 26 753 (1932)

³²³ R. A. Peters and R. H. S. Thompson *Ibid* 28 916 (1934)

³²⁴ E. Bueding and H. Wortis *J Biol Chem* 133 585 (1940)

³²⁵ M. Shils, H. G. Day and E. V. McCollum *Ibid* 139 145 (1941)

function of the type and quantity of food taken in. The urinary output of pyruvic acid appears to be higher in the male than in the female.

Blood Tests The determination of vitamin B_1 in plasma is rather difficult since the concentration is normally very small, about 1 γ in 100 ml. Furthermore slight hemolysis of the blood affects the results considerably.³²⁵ The only method therefore which has been found useful is the determination of the growth rate of *Phycomyces blakesleanus*.³²⁷ Blood contains however, other factors which increase the growth rate of this fungus. Nevertheless the method is valuable for comparing the apparent vitamin B_1 content in different samples of blood.³²⁸ The difficulties can partly be overcome by evaluation of the growth rate by blood with and without the addition of vitamin B_1 .

The determination of vitamin B_1 pyrophosphate (cocarboxylase)^{326, 329, 330, 331} can be carried out much more easily. The pyrophosphate occurs exclusively in the blood cells. Therefore in cases in which the blood cell count is disturbed for example in cases of polycythemia or of myeloid leukemia the cocarboxylase determination should not be used. The method consists in the determination of the carbon dioxide production from pyruvic acid in the presence of yeast as a source of carboxylase and in the presence of excess amounts of vitamin B_1 . Instead of using whole blood for the determination the use of washed blood cells has been recommended. Blood of healthy adults contains an average of about 7 γ of cocarboxylase per 100 ml. A value below 3 γ is considered as indicative of a state of vitamin B_1 deficiency.

The determination of pyruvic acid is carried out as described for its determination in urine. A marked increase in pyruvic acid has been found in man and in experimental animals, such as in pigeons³³ and in rats³³³ during times of vitamin B_1 deficiency. The normal pyruvic acid content of human blood averages 2.8 mg per 100 cc.

17 Hypervitaminosis

Vitamin B_1 has no specific pharmacological action as far as is known to day. The vitamin is non toxic even in doses which are several thousand times larger than a normal daily dose. No cases of human vitamin B_1

³²⁵ R. Goodhart and H. M. Sinclair *Biochem J.* 33, 1099 (1939).

³²⁶ H. M. Sinclair *Ibid.* 33, 2027 (1939).

³²⁷ H. M. Sinclair *Ibid.* 32, 185 (1938).

³²⁸ S. Ochoa and R. A. Peters *Ibid.* 32, 1501 (1938).

³²⁹ R. Goodhart and H. M. Sinclair *J. Biol. Chem.* 132, 11 (1940).

³³⁰ R. Goodhart *Ibid.* 133, 77 (1940).

³³¹ R. H. S. Thompson and R. E. Johnson *Biochem J.* 29, 694 (1935).

³³ C. D. Lee *Ibid.*, 83, 774 (1939).

hypervitaminosis have ever been reported. The therapeutic index, that is, the ratio of the therapeutic dose to the minimum lethal dose, is extremely high: 600 for mice, 5000 for rats and 70,000 for dogs. Doses of 125 mg per kg body weight for mice, 250 mg per kg for rats, 300 mg per kg for rabbits, or 350 mg per kg for dogs are lethal when injected intravenously.³³⁴

18 Requirements

It is impossible to define accurately the requirements of vitamin B₁. The amount needed is not a constant, but a function of the supplied food, of the intensity of the metabolism, of the outside temperature and of other factors. The consumption of carbohydrates increases and of fat decreases the amount of vitamin B₁ needed by the organism, while proteins do not have any influence on the vitamin requirement. The intensity of the total metabolism is again a function of many single factors, among which might be mentioned the secretion of thyroxin, the physical labor performed, pregnancy, etc.

The ratio of the minimum amount of vitamin B₁ needed to support life to the optimal amount for general development of the body is very high and can vary from 3 up to 100.

The vitamin B₁ requirement of man therefore cannot be defined exactly. The allowances as recommended by the Food and Nutrition Board of the National Research Council (for details see page 613) call for a daily intake varying from 1.2–2.3 mg of thiamin for adults depending upon sex and relative activity. The requirements for children are correspondingly lower. A six to eight months old baby should receive 0.4 mg of thiamin.³³⁵

Vitamin B₁ is as far as is known needed by all animals even by insects and microorganisms. Sheep³³⁶ and cattle^{337, 338, 339} apparently need no external supply of this vitamin, since it is synthesized by bacteria in the rumen of these animals.

³³⁴ H. Molitor, *Merck's Jahresberichte* 1936, 51.

³³⁵ E. M. Knott, *Proc. Soc. Exptl. Biol. Med.* 45, 765 (1940).

³³⁶ L. W. McElroy and H. Goss, *J. Biol. Chem.* 130, 437 (1939).

³³⁷ L. W. McElroy and H. Goss, *Ibid.* 133, LXV (1940).

³³⁸ M. I. Wegner, A. N. Booth, C. A. Elvehjem and R. B. Hart, *Proc. Soc. Exptl. Biol. Med.* 45, 769 (1940).

³³⁹ S. I. Bechdel, H. E. Honeywell, R. A. Dutcher and M. H. Knutsen, *J. Biol. Chem.*, 80, 231 (1928).

• VITAMIN B₂—
RIBOFLAVIN

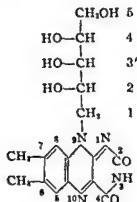
VITAMIN B₂—RIBOFLAVIN

1 Nomenclature and Survey

Names

- Riboflavin name used in America¹
 Lactoflavin name used in Europe
 Ovoflavin and hepatoflavin historical names indicating the origin
 Vitamin G historical designation by Sherman

Chemical formula



Chemical name

- 6,7 Dimethyl 9-(d-1' ribityl) iso-alloxazine
 6,7 Dimethyl 9-d riboflavin

Empirical formula



Efficacy

1 g riboflavin = 400 000 Bourquin-Sherman Units

Classification

Vitamin B₂ belongs to the class of colored water soluble naturally occurring substances which carry the group name lyochromes. In distinction from the lyochromes the colored fat or organic solvent soluble naturally occurring substances

¹ The term *d* riboflavin or just riboflavin was suggested by the Council on Pharmacy and Chemistry, *J Am Med Assoc* 108:1340 (1937) and approved by the Committee on Vitamin Standards, American Society of Biological Chemists and also by the Committee on Vitamin Nomenclature, American Institute of Nutrition.

are called *lyochromes*. The lyochromes are not as yet systematically subclassified. Arbitrarily vitamin B₂ is a member of the subdivision flavins. The members of this group carry names indicating the origin of their occurrence. Thus lactoflavin comes from milk, ovoflavin from eggs, hepatoflavin from liver, uroflavin from urine, etc. All naturally occurring flavins which have been investigated with the exception of uroflavin proved to be identical with riboflavin.

2 Chronology

- 1879 BLYTH¹ isolated an impure flavin from whey
 1913 OSBORNE and MENDEL recognized in milk the presence of a water soluble substance which promotes growth.²
 1917 EMMETT and MCKIN differentiated the physiological action of vitamin B₁ from vitamin B₂.³
 1925 BLEYER and KALLMANN obtained a yellow pigment called lactochrome in crude form from milk.⁴
 1932 WARBURG and CHRISTIAN isolated the yellow enzyme from yeast.⁵ BANGA and SZENT GYÖRGY⁷ recognized a respiration co ferment in yeast the colored part of which was called cytoflav.
 1933 ELLINGER and KOSCHARA and KUHN, GYÖRGY and WAGNER JAUREGG isolated pure riboflavin. The latter authors recognized the identity of vitamin B₂ and riboflavin.⁶
 1935 KUHN and KARRER and their groups established the constitution of riboflavin by total synthesis.

3 Occurrence

Riboflavin is very widely distributed over the entire animal and plant kingdom.⁸ It seems that each animal and plant cell contains small amounts. The amount in plant seeds is small but increases rapidly during germination. The richest source of riboflavin is anaerobic growing fermentation bacteria, for example butyric acid bacteria in the dried state contain up to 15 mg %. Also various yeasts contain fair amounts of riboflavin. Liver, kidney and heart of vertebrata and fish livers contain about 10–30 mg % or about 10–30 times more than muscles. The retina

¹ A. W. Blyth, *J. Chem. Soc.* 35, 330 (1879).

² T. B. Osborne and L. B. Mendel, *J. Biol. Chem.* 15, 311 (1913).

³ A. D. Emmett and L. H. McKin, *Ibid.* 32, 409 (1917).

⁴ B. Bleyer and O. Kallmann, *Biochem. Z.* 155, 54 (1925).

⁵ O. Warburg and W. Christian, *Naturwissenschaften* 20, 688, 980 (1932); *Biochem. Z.* 254, 438 (1932); 257, 492 (1933); 266, 377 (1933).

⁷ I. Banga and A. Szent György,

203 (1932).

Szent György and

I. Vargha, *Z. phys. Chem.* 210, 2.

physiol. Chem.

H. v. Euler and

⁸ P. György, R. Kuhn and T. W. J. Adler, *Ibid.* 223, 105 (1934).

of the eyes of many species of animals contains considerable quantities of riboflavin⁹

Vitamin B₂ occurs in the animal organism in a number of different forms. In milk, in urine¹⁰ and in the retina,¹¹ or, generally speaking, in places where no respiration or fermentation takes place the free riboflavin is found. In tissues riboflavin occurs as such as riboflavin phosphoric acid and in the form of riboflavin phosphoric acid adenine dinucleotide. Each of these forms occurs in the free state as well as combined with specific proteins (see page 171). For a combination of riboflavin with protein in muscles, see¹²

4 Isolation

The isolation of riboflavin is carried out by extraction of flavin containing materials with aqueous acid solutions with water alcohol mixtures with alcohol or with acetone. Direct extraction of natural materials especially of plant origin yields only part of the total riboflavin present. The remaining vitamin is chemically bound but can be liberated by heating.¹³ The extraction is followed by a combination of different precipitation and adsorption procedures. The following precipitation methods have been used:^{14, 15} lead acetate, phosphotungstic acid in normal sulfuric acid followed by extraction of the precipitate with amyl alcohol, silver nitrate or mercuric sulfate in acid solution precipitates other substances leaving the vitamin B₂ in solution but silver nitrate in neutral solution precipitates the vitamin. By the addition of alcohol to concentrated aqueous riboflavin solutions salts and glycogen are eliminated.

Riboflavin is adsorbed in acid solution by fuller's earth¹⁶ or in neutral solution by frankonit. Charcoal can also be used successfully. Talc, aluminum oxide, calcium carbonate, kaolin and kieselguhr do not absorb vitamin B₂. The elution is carried out by basic solvents such as pyridine.

⁹ O. Brunner and E. Baron, *Monatsh.* 68, 764 (1936).

¹⁰ A. F. Emmerie, *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.* 8, 116 (1938).

¹¹ H. v. Euler and E. Adler, *A. E. v. Keme Mineral. Geol.* B11, No. 28 (1934). R. Kuhn and H. Kalt, *Schmitt Ber.* 68, 396 (1933). E. Adler and H. v. Euler, *Nature* 141, 790 (1938). H. v. Euler and E. Adler, *Z. physiol. Chem.* 223, 100 (1934).

¹² J. Schormüller, *Z. Untersuch. Lebensm.* 77, 1 (1933).

¹³ F. M. Lantz, *Ag. Exptl. Sta. New Mexico Coll. Ag. Mech. Arts Bull.* 268 (1933).

¹⁴ B. C. Cuba, *Biochem. J.* 25, 940 (1931).

¹⁵ P. György, R. Kuhn and T. Wagner-Jauregg, *Z. physiol. Chem.* 223, 21, 27, 36, 241 (1934).

¹⁶ A. Seidel, *U. S. Ind. Health Serv. & Ind. Health Reps.* 31, 404 (1916). W. D. Salmon, N. B. Cuernant and I. M. Hays, *J. Biol. Chem.* 80, 61 (1928). B. T. Narayanan and J. C. Drummond, *Biochem. J.* 24, 19 (1930).

methanol water mixtures,^{17 18} ammonia, caustic solutions, triethanolamine or by neutral organic solvents, such as 80% acetone. After precipitation, together with lead sulfide, riboflavin is extracted with hot water.^{17 19}

From concentrates obtained from the elution of adsorbates, riboflavin usually crystallizes out in clusters of fine orange yellow needles. They can be purified further by precipitation with thallium ions or by one of the previously described precipitation methods. Final recrystallizations are carried out from water, aqueous alcohol or diluted acetic acid.

By such methods, the pure vitamin B₂ has been obtained by many workers,^{20 21} and the identity of the flavins from different sources has been established chemically and biologically. Ovoflavin from egg white, lactoflavin from milk, hepatoflavin from liver, etc., are identical with riboflavin. One gram of riboflavin was obtained from 5400 liters of milk.

Methods for the separation of vitamin B₁ from vitamin B₂ are discussed on page 102. It might be added here that vitamin B₁ can be destroyed by heating to 120° C for six hours, leaving the vitamin B₂ mainly intact.²⁰

For methods of separating riboflavin, riboflavin phosphoric acid and the flavin enzymes from each other see page 183.

5 Properties

Riboflavin crystallizes in fine orange yellow needles which melt at 282° C under decomposition (darkening at about 240°). The pure compound is slightly soluble in water (12 mg in 100 cc at 27.5°, 19 mg at 40°) and in ethyl alcohol (4.5 mg at 27.5°), amyl alcohol, cyclohexanol, phenol, amyl acetate, etc., and is very soluble in alkali solutions. It is insoluble in acetone, ether, benzene and chloroform. The impure material is much

¹⁷ P. Ellinger and W. Koschka *Ber* 66 315 808 1411 (1933)

¹⁸ R. Kuhn, P. György and T. Wagner Jauregg *Ibid* 66 317 571 1034 1577 (1933) *Naturwissenschaften* 21 560 (1933) *Klin Wochschr* 12 1241 (1933)

¹⁹ P. Karrer, H. Salomon and K. Schöpp *Helv Chim Acta* 17 419 735 (1934)

²⁰ P. Ellinger and W. Koschka *Ber* 66 315 808 1411 (1933)

²¹ R. Kuhn, P. György and T. Wagner Jauregg *Ibid* 66 317 576 1034 1577 (1933) *Naturwissenschaften* 21 560 (1933) *Klin Wochschr* 12 1241 (1933)

²² P. Karrer, H. Salomon and K. Schöpp *Helv Chim Acta* 17 419 735 (1934)

²³ L. E. Booher *J Biol Chem* 102 39 (1933) 107 591 (1934)

²⁴ S. Lepkovsky, W. Popper and H. M. Evans *Ibid* 108 257 (1935) 109 *Proc* 54 (1935)

²⁵ S. Itter, E. R. Orent and E. V. McCollum *Ibid* 108 579 (1935)

²⁶ C. A. Elvehjem and C. J. Koehn *Ibid* 108 709 (1935)

²⁷ F. J. Stare *Ibid* 111 567 (1935)

²⁸ S. Ansbacher, G. C. Supplee and R. C. Bender *J Nutrition* 11 401 (1936)

²⁹ R. D. Greene and A. Black *J Am Chem Soc* 59 1820 (1937)

³⁰ M. I. Smith and L. G. Hendrick *U S Pub Health Service Pub Health Repts* 41 201 (1926)

A. Seidel *Bull soc chim biol* 8 746 (1926) A. Haas and J. C. Drummond *Biochem J* 21 653 (1927) H. Chick and M. H. Roscoe *Ibid* 21 648 (1927) H. C. Sherman and J. H. Atmayer *J Biol Chem* 75 107 (1927)

more soluble than the pure material. The water solution is of greenish yellow color and displays an intense yellow green fluorescence which vanishes on the addition of acids. Alkali also causes disappearance of the fluorescence by shifting the hydrogen from the amino group in 3 position to the neighboring oxo group, thus forming an enol. The fluorescence (maximum at 565 m μ) is used for the quantitative determination of riboflavin (see page 182). Optimum fluorescence occurs at pH 3 to 9. The isoelectric point of vitamin B₂ is at pH 6. Riboflavin is thus

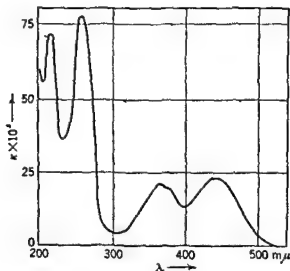


Fig. 10—Absorption spectrum of vitamin B₂ (riboflavin) (R. Kuhn)

amphoteric in character. The isoelectric constants for acid and base are calculated to be ²¹ $K_{\text{acid}} = 6.3 \times 10^{-12}$ and $K_{\text{base}} = 0.5 \times 10^{-3}$. Riboflavin shows optical rotation $[\alpha]_D^{20} = -114^\circ$ in 0.1 normal sodium hydroxide ²². In neutral and in acid solution, the optical activity is exceedingly small. Riboflavin has a characteristic absorption spectrum with maxima at 445, 372, 269 and 225 m μ (Fig. 10).

Crystalline riboflavin, when protected against light, is stable at ordinary temperatures. Light slowly destroys the vitamin activity. In solution vitamin B₂ is essentially unstable. The decomposition is greatly influenced by light, temperature and pH of the solution. Under alkaline

²¹ R. Kuhn and G. Moruzzi *Ber.* 67 888 (1914).

²² R. Kuhn and H. Ridy *ibid.* 68 160 (1915). P. Karrer and H. L. L. *Helv. Chim. Acta* 18 1174 (1935).

conditions riboflavin decomposes rapidly. Riboflavin possesses a relatively high degree of thermostability; thus only slight destruction occurs by heating to 120° C for six hours.

6 Chemical Constitution of Vitamin B₂ Degradation Reactions

Riboflavin has the empirical formula C₁₇H₂₀N₄O₆. It is resistant against acids, bromine and oxidizing agents such as hydrogen peroxide and concentrated nitric acid. Chromic acid oxidizes the molecule to ammonia, carbon dioxide and a nitrogen free residue of unknown constitution. Acetylation of riboflavin yields a tetra acetate, indicating the presence of four hydroxyl groups.^{22, 24} A diacetone compound can be formed, which indicates the close position of each two hydroxyl groups.²⁵ Oxidation of riboflavin with lead tetra acetate yields 0.8 mol of formaldehyde. A primary hydroxyl group is, therefore, in α position to a secondary hydroxyl group.²⁴ Primary amino groups are not present since nitrous acid does not affect riboflavin. Alkaline hydrolysis yields urea²³ indicating the presence of the configuration —NH—CO—NH—. The other two nitrogen atoms are tertiary. Irradiation destroys vitamin B₂. Irradiation in alkaline solution yields a new compound, called lumiflavin (lumi lacto flavin) or photoflavin and a sugar compound C₄H₈O₄, which could not be isolated as such, probably because of decomposition. Lumiflavin cannot be acetylated further and does not give formaldehyde by oxidation with lead tetra acetate. Vitamin B₂, therefore, contains a side chain of the constitution of a tetrahydroxy butyl group,



the configuration of which could only be determined by total synthesis of riboflavin. It proved to be *d* ribose.

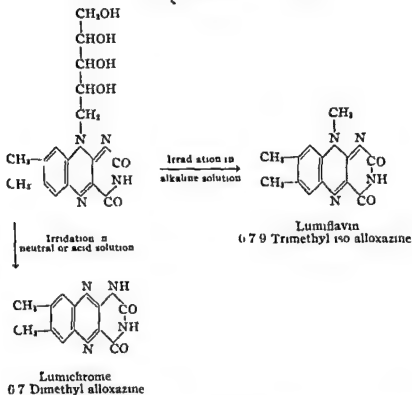
Photolysis of riboflavin in neutral solution *in vacuo* causes the disappearance of the yellow color with the formation of *deutero leuco riboflavin* which can be dehydrogenated by oxygen. Alkali causes the dehydrogenated compound to be converted into the already mentioned lumiflavin.²⁶

²² R. Kuhn and T. Wagner Jauregg *Ber.* 66 1577 (1933)

²³ R. Kuhn, H. Rudy and T. Wagner Jauregg *Ibid.* 66 1950 (1933)

²⁴ R. Kuhn, H. Rudy and F. Weygand *Ibid.* 68 62 (1935)

²⁵ R. Kuhn, H. Rudy and T. Wagner Jauregg *Ibid.* 66 1950 (1933)

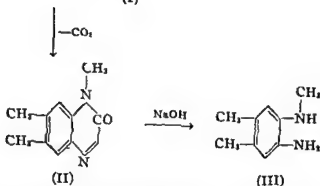
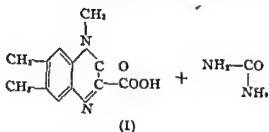


Lumiflavin contains a methyl group¹⁷ which was not present in riboflavin and which, therefore, must have replaced the missing sugar side chain. The hydroxyl groups of the side chain are therefore connected with the flavin ring system by a methylene group. Alkaline hydrolysis of lumiflavin yields urea and an oxo carboxylic acid, C₁₂H₁₁N₂O₃^{18, 19} (I), which contains one active hydrogen atom and exhibits the properties of a monobasic acid upon titration. Since two molecules of water are required for the alkaline hydrolysis of lumiflavin the urea must come from a ring system and not from a side chain ureide or guandino group which would require only one molecule of water for hydrolysis. The oxo acid (I) is decarboxylated upon heating yielding the lactam (II). By heating the lactam with sodium hydroxide, 1,2 dimethyl-4 amino 5 methyl amino benzene (III) is formed. This *o* phenylene-diamine gives a bluish green color reaction with ferric chloride, which is, according to Noelting, characteristic for *p,p*-disubstituted *o* phenylene-diamines.

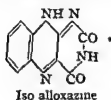
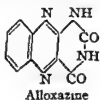
¹⁷ R. Kuhn and H. Rudy *Ber.* 67 1798 (1934)

¹⁸ R. Kuhn and T. Wagner-Jauregg *Ibid.* 66 1577 (1917)

¹⁹ R. Kuhn and H. Rudy *Ibid.* 67 897 (1934)



Irradiation of riboflavin yields lumiflavin in alkaline solution and lumichrome, in neutral or in acid solution⁴⁰ Lumichrome has the constitution of 6,7-dimethyl alloxazine, and is an intensely fluorescent compound. It is a derivative of alloxazine as proved by synthesis, whereas lumiflavin and riboflavin are derivatives of the hypothetical iso alloxazine. Lumiflavin and riboflavin have one active hydrogen atom in 3 position; lumichrome contains active hydrogens in both 1- and 3 positions.



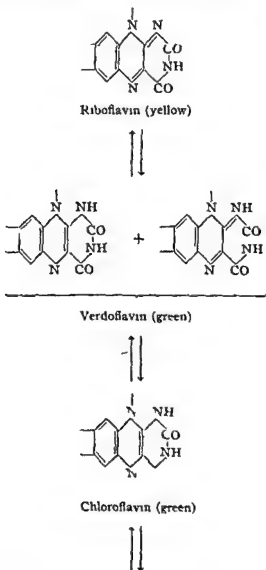
Riboflavin readily takes up two hydrogen atoms⁴¹. It is reversibly reduced by hydrogen in the presence of a catalyst, by zinc in acid solution, by sodium thiosulfate, by hydrogen sulfide in alkaline solution and by titanous chloride. The dihydro compound is colorless, shows no fluorescence and is called leuco riboflavin (leuco lactoflavin). The leuco compound is easily oxidized to riboflavin by air. The redox potential of an equimolecular mixture of riboflavin and its leuco compound is -0.21 volts.⁴²

⁴⁰ P. Karrer, H. Salomon, K. Schöpp, F. Schlatter and H. Fritzsche, *Helv. Chim. Acta* 17, 1010 (1934).

⁴¹ R. Kuhn, P. György and T. Wagner-Jauregg, *Ber.* 66, 576 (1933).

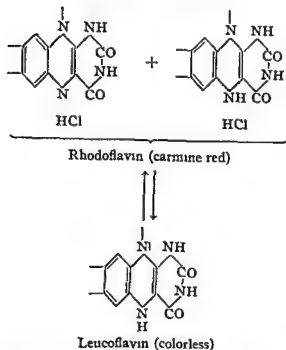
⁴² L. S. G. Barron and A. B. Hastings, *J. Biol. Chem.* 105, VII (1934); L. Bierich and A. Lang, *Z. physiol. Chem.* 223, 180 (1934); K. C. Stern, *Biochem. J.* 28, 94J (1934); R. Kuhn and G. Moruzzi, *Ber.* 67, 1720 (1934); R. Kuhn and P. Boulanger, *Ibid.* 69, 1557 (1936); P. J. Stare, *J. Biol. Chem.* 112, 273 (1935).

It has been postulated that by reduction of riboflavin to the leuco compound, three intermediate compounds result, which consist of molecular compounds of reduced and unreduced molecules.⁴³ *Verdoflavin* consists of riboflavin and monohydro riboflavin, *chloroflavin* is a quinhydrone of riboflavin and leuco riboflavin, and *rhodoflavin* hydrochloride contains the hydrochlorides of leuco riboflavin and monohydro riboflavin



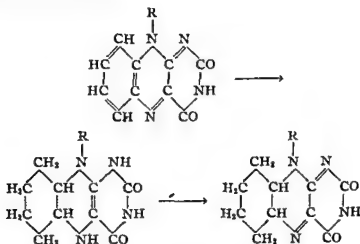
(Formula continued on follow page)

⁴³ R. Kuhn and R. Ströbel, *Ber.* 70 753 (1937)



The existence of these intermediate stages is somewhat dubious, since titration curves of the reduction process indicate the presence of only one intermediate⁴⁴. Since the quinhydrone is the only form that exists in *diluted* aqueous solution, it seems reasonable to assume that only this form occurs under physiological conditions.

By energetic catalytic hydrogenation of flavins, octahydro flavins are obtained⁴⁵ which are easily oxidized in alkaline solution by air to the corresponding hexahydro flavins.

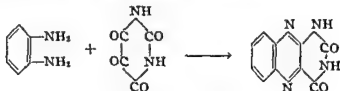


⁴⁴ L. Michaelis and G. Schwarzenbach *J Biol Chem* 123 577 (1938)

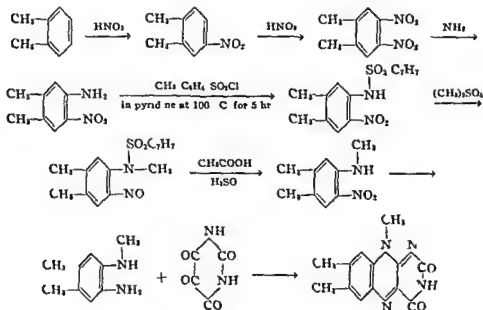
⁴⁵ P. Karrer and R. Ostwald *Rec trav chim* 57 500 (1938)

7 Synthesis of Vitamin B₂ and Other Flavins

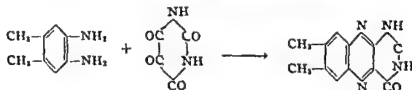
Alloxazines were first synthesized by Kuhling⁴⁶ by condensation of the hydrochloride of *o* phenylene diamine with alloxan



Kuhn and co-workers⁴⁷ synthesized lumiflavin from alloxan and 1,2 dimethyl-4 amino 5 methyl amino benzene in acid solution. The *N* methyl *o* xyllylene diamine was obtained according to the following scheme



Karrer and co workers⁴⁸ synthesized lumichrome similarly

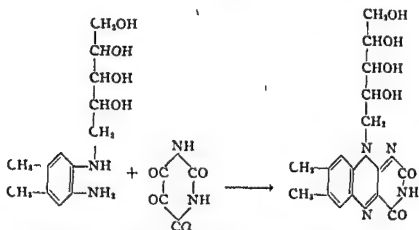


⁴⁶ O. Kuhling, *g Ber* 24 2363 (1891) 27 1116 (1894) 28 1968 (1895) O. Kuhling and O. Kasitz, *Ibid* 39 1314 (1906)

⁴⁷ R. Kuhn, K. Reinemund and F. Weygand, *Ibid* 67 1460 (1934) R. Kuhn and K. Reinemund, *Ibid* 67 1932 (1934) R. Kuhn, H. Rudy and K. Reinemund, *Ibid* 68, 170 (1935)

⁴⁸ F. Karrer, H. Salomon, K. Schöpp, F. Schlatter and H. Fritzche, *Helv Chim Acta* 17 1010 (1934)

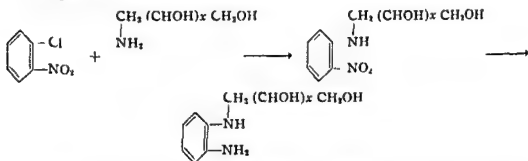
According to the same principle, riboflavin is obtained from alloxan and *di* methyl amino phenyl ribamine



The condensation of the 4,5 dimethyl 2 amino phenyl ribamine with alloxan is carried out in acid solution. When boric acid is used as catalyst in acetic acid solution the yield of the condensation product increases considerably.⁴⁹

The synthesis of the dimethyl amino phenyl ribamine has been achieved by the following methods

1 *o* Nitro chloro benzenes are condensed with amino sugars and the reaction product hydrogenated to the diamine.⁵⁰ This method gives satisfactory yields when the sugar compound contains two or three hydroxyl groups, but poor yields with sugars containing four and five hydroxyl groups



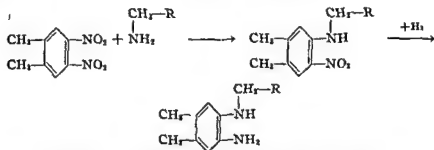
2 *o* Dinitro xylene is condensed with ribamine in aqueous alcoholic solution and catalytically reduced to the corresponding diamine.⁵¹ The

⁴⁹ R. Kuhn and F. Weygand *Ber.* 68 1982 (1935). See also R. Kuhn and A. H. Cook *Angew. Chem.* 49 6 (1936).

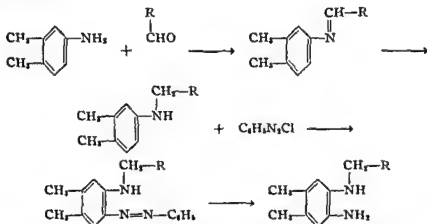
⁵⁰ P. Karrer, H. Salomon, L. Schöpp and I. Schlitter *Helv. Chim. Acta* 17 1516 (1934). I. Karrer and I. Schlitter, K. Pfäffler and F. Benz *Ibid.* 17 1516 (1934).

⁵¹ R. Kuhn and F. Weygand *Ber.* 68 1001 (1935).

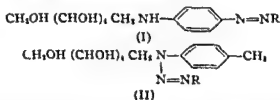
over all yield of riboflavin according to this process is 4.5% of the ribose used



3.3.4 Xylidene⁴² is condensed with ribose and the formed riboside is catalytically reduced. The second amino group is introduced by coupling with diazonium salts to form azo dyes which by reduction yield the desired diamine.⁴³



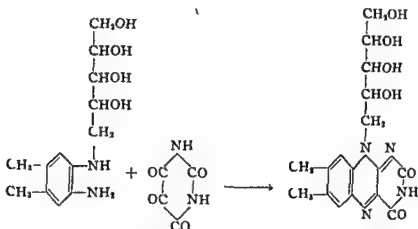
This method gives very high yields on riboflavin (38%) calculated from the required ribose but cannot be used for the preparation of any other flavin. Only *m*, *p* disubstituted aniline derivatives couple with diazonium salts in *o* position. Phenyl glucamine for example couples in *p* position, yielding (I). *p* toluene glucamine yields (II).



⁴² P. Karrer, B. Becker, F. Benz, P. Frey, H. Salomon and K. Schöpp, *Helv. Chim. Acta* **18**, 1435 (1935); W. A. Witsensky and S. Ansbacher, *J. Am. Chem. Soc.* **63**, 2532 (1941).

⁴³ P. Karrer and H. Moerwede, *Helv. Chim. Acta* **18**, 1170 (1935); **19**, 264 (1936).

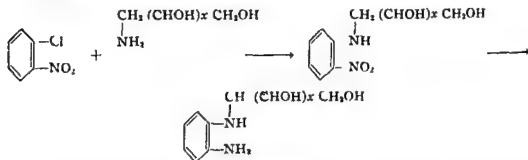
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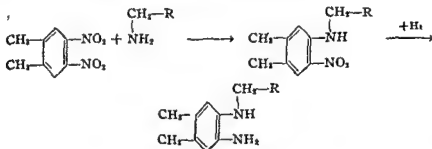
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⁴⁹ R. Kuhn and F. Weygand *Ber.* 68 1982 (1935). See also R. Kuhn and A. H. Cook *Angew. Chem.* 49 1 (1936).

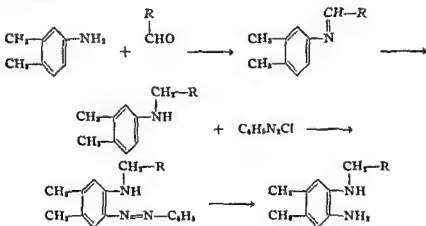
⁵⁰ P. Karrer, H. Salomon, K. Schöpp and I. Schlitter *Helv. Chim. Acta* 17 110 (1934). I. Karrer and I. Schlitter, K. Pfäffler and F. Benz *Ibid.* 17 1 16 (1934).

⁵¹ R. Kuhn and F. Weygand *Ber.* 68 1001 (1935).

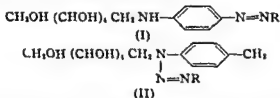
over all yield of riboflavin according to this process is 4.5% of the ribose used



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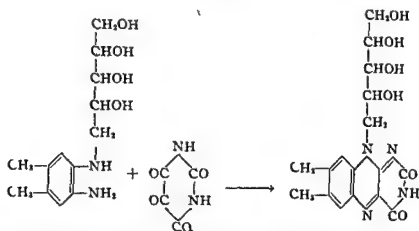
This method gives very high yields on riboflavin (38%) calculated from the required ribose but cannot be used for the preparation of any other flavin. Only *m*, *p* disubstituted aniline derivatives couple with diazonium salts in *o* position. Phenyl glucamine, for example couples in *p* position, yielding (I). *p*-toluene-glucamine yields (II).



³¹ P. Karrer, B. Becker, F. Henz, I. Frei, H. Salomon, and A. Schöpp, *Helv. Chim. Acta* **18**, 1435 (1935); W. A. Witsky and A. Ausbacher, *J. Am. Chem. Soc.* **63**, 2532 (1941).

³² P. Karrer and H. Meerwein, *Helv. Chim. Acta* **18**, 1150 (1935); **19**, 264 (1936).

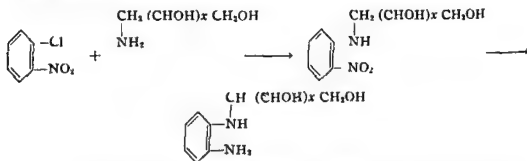
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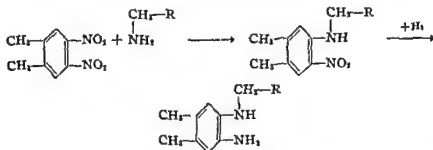
2 *o* Dinitro xylene is condensed with ribamine in aqueous alcoholic solution and catalytically reduced to the corresponding diamine.¹ The

⁴⁹ R. Kuhn and F. Weygand *Ber.* 68 1282 (1935). See also R. Kuhn and A. H. Cook *Angew. Chem.* 49 f (1936).

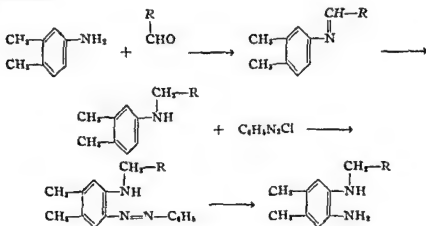
⁵⁰ P. Farrer, H. Salomon, K. Schöpp and F. Schlatter *Helv. Chim. Acta* 17 1115 (1934). P. Farrer, F. Schlatter, K. Pfäehler and F. Benz *Ibid.* 17 1116 (1934).

⁵¹ R. Kuhn and F. Weygand *Ber.* 68 1001 (1935).

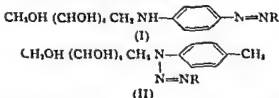
over all yield of riboflavin according to this process is 4.5% of the ribose used



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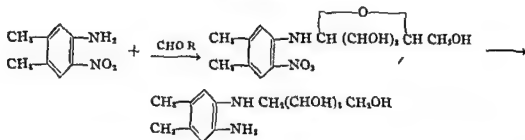
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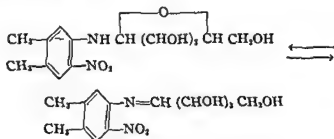
⁵² P. Karrer, B. Beck, F. Heus, P. Frei, H. Salomon and K. Schöpp *Helv. Chim. Acta* 18 1433 (1935); W. A. Witsansky and N. Ansbacher *J. Am. Chem. Soc.* 63 2532 (1941).

⁵³ P. Karrer and H. Meerwein *Helv. Chim. Acta* 18 1150 (1935); 19 264 (1936).

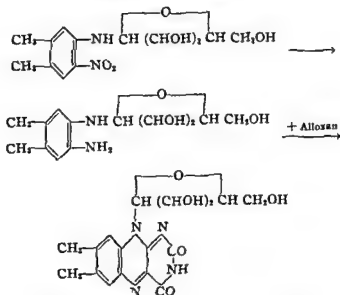
4 *o* Nitro xylidine is condensed with ribose and the reaction product is catalytically reduced to the diamine.⁵⁴ This method yields 16% riboflavin calculated on the amount of ribose used



The intermediate is an amino glucoside which exists in an equilibrium with the tautomeric Schiff base



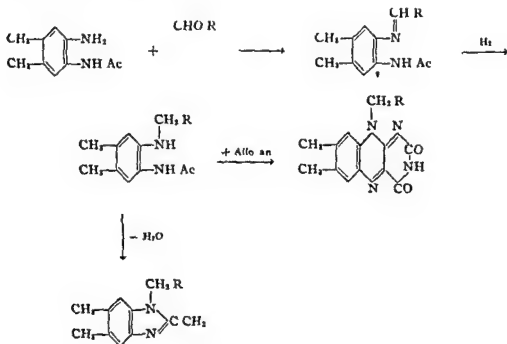
The Schiff base can also be reduced partially to the *o* phenylene diamine glucoside which after acetylation of the free hydroxyl groups may be con



⁵⁴ R. Kuhn and R. Ströbele *Ber* 70 773 (1937) R. Kuhn, K. Reinemund, J. Weygand and R. Ströbele *Ibid* 68 176 (1935)

densed with alloxan to the corresponding acetylated flavin glucoside⁴⁵ The latter upon saponification yields the free glucoside Flavin glucosides do not exhibit the physiological properties of vitamin B₂

5 *N* Mono acyl *o* phenylene diamines condense with sugars to the Schiff bases which are reduced simultaneously to the *N* (*o* acyl amino phenyl) amino sugars⁴⁶ These are condensed in acid solution with alloxan to the corresponding flavins The acyl groups are automatically saponified during the reaction The yields on flavins according to this process are extremely low, since the main reaction is an intermolecular dehydration which yields benzimidazole derivatives

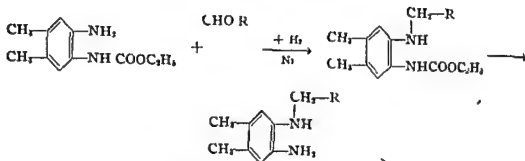


6 In the preceding method one amino group is temporarily protected by acetylation while the other amino group is condensed with ribose A better method is the protection of the amino group by the carbethoxy group⁴⁷

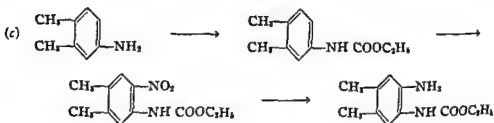
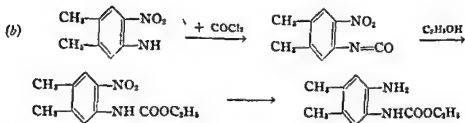
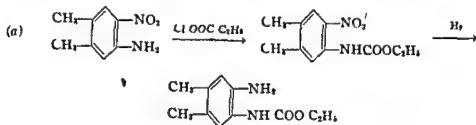
⁴⁵ R. Fuhs and R. Ströbele *Ber* 70 74 (1937)

⁴⁶ P. Karrer, K. Schöpp, F. Benz and K. Pfeiffer *Helv Chim Acta* 18 69 (1935) *Ber* 68 216 (1935)

⁴⁷ P. Karrer, K. Schöpp, F. Benz and K. Pfeiffer *Helv Chim Acta* 18 69 (1935) P. Karrer, K. Schöpp and F. Benz *Ibid* 18 426 (1935) H. v. Euler, P. Karrer, H. Malmberg, K. Schöpp, F. Benz, R. Beck and F. Frei *Ibid* 18 2 (1935) P. Karrer, H. Salomon, K. Schöpp, F. Benz and R. Becker *Ibid* 18 908, 1143, 1435 (1935) P. Karrer and F. M. Strog *Ibid* 18 1343 (1935)



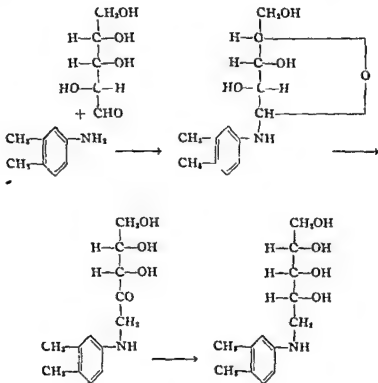
The urethane can be prepared by one of the following procedures



The yield of riboflavin according to this procedure is approximately 14-15% of the ribose used

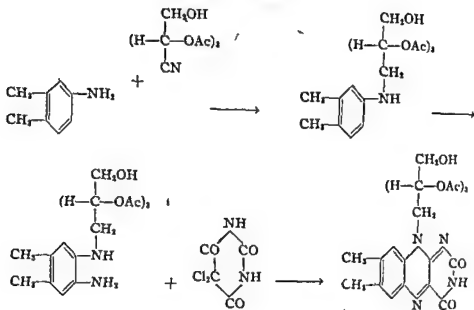
7 While in the procedures described under (1 to 6) *d* ribose has been used as an intermediate, it is also possible to use some other sugar derivatives. A very elegant method^{66a} for the preparation of dimethyl amino phenyl ribamine is to condense 3,4 xylidine with *d* arabinose in the presence of small amounts of acid to form the *d* arabinoside which upon heat

ing to 75° C undergoes an Amadori rearrangement to the *d* iso arabinosamine. Upon catalytic hydrogenation in alkaline solution the *d* iso arabinosamine is converted into 3,4 dimethyl phenyl *d* ribamine. This compound is obtained in about 13% of theory from the arabinose originally used. The arabamine is then coupled with a diazonium salt and reduced to the desired diamine as described under (3).



8 3,4 Xylidine is condensed^{46b} reductively with tetra acetyl ribono nitrile which is obtained from ribonic acid via the amide. The dimethyl phenyl ribamine is then coupled with para nitrophenyl diazonium chloride and reduced to N tetra acetyl ribitylamino 2 amino-4,5 dimethyl benzene according to the procedure described under (3). It has been recommended to condense the last mentioned compound with 5,5' dichloro barbituric acid to obtain vitamin B₂ instead of using alloxan as has been described previously.

^{46b} M. Tihler and J. W. Wellman, U. S. 2,611,008



8 Industrial Methods of Preparation

The relative technical importance of natural *versus* synthetic vitamin B₂ is dependent on the use for which the vitamin is intended. For animal foods, various riboflavin preparations from natural sources such as yeast, whey or anaerobic growing fermentation bacteria, for example, from butyl alcohol fermentations are used. The riboflavin content of these preparations varies considerably and must be standardized. For human therapy, the use of pure riboflavin is preferred. Since the isolation of pure riboflavin is more expensive than the synthesis thereof, synthetic vitamin B₂ is generally used clinically, especially in cases of an exclusive vitamin B₂ deficiency.

The synthesis of vitamin B₂ is carried out according to the methods described in the preceding section. The starting materials are *o*-xylene, *d*-ribose and alloxan. *o*-Xylene is available as a by-product from the petroleum industry. *d*-Ribose is obtained either from natural sources or by synthesis. A convenient method for the preparation of small amounts of *d*-ribose is the hydrolysis of yeast nucleic acid.⁵⁸ Synthetic *d*-ribose is prepared from *d*-glucose.^{59, 60} Alloxan is usually obtained by oxidation of uric acid or barbituric acid.

⁵⁸ H. Brederick, *Ber.* 71, 408 (1938); H. Brederick, M. Köhnig and F. Berger, *Ib. id.* 73, 936 (1940).

⁵⁹ W. C. Autin and F. I. Hummoller, *J. Am. Chem. Soc.* 56, 1152 (1934).

⁶⁰ R. Kuhn, K. Reinemund, F. Weygand and R. Ströbele, *Ber.* 68, 1765 (1935).

9 Flavin-Enzymes

(a) Enzyme Systems Containing Riboflavin

Riboflavin takes part in a number of different enzyme systems in tissues. These systems consist of an apoenzyme and a coenzyme. The apoenzyme is a specific protein and is also called pherion bearer protein or Zwischenferment. The coenzyme constitutes the prosthetic group of the enzyme system and contains riboflavin as part of its constitution. There are two different riboflavin-containing coenzymes namely a mononucleotide and a dinucleotide. The mononucleotide has the constitution of a riboflavin phosphate while the dinucleotide is a riboflavin adenine dinucleotide. (For details see page 177) These coenzymes combine with different apoenzymes to carry out specific reactions. The same coenzyme can serve as the prosthetic group of a number of different apoenzymes. The function of all enzyme systems is to transfer hydrogen for example in the carbohydrate and amino acid metabolism. In the carbohydrate metabolism for example the substrate is oxidized by dehydrogenation through the nicotinamide containing enzymes which are reduced in this process to the dihydro compounds which in turn are oxidized by the riboflavin containing enzymes. Thus the dihydro nicotinamide enzymes serve as substrates for the riboflavin enzymes which are converted into dihydro derivatives (see page 180). The dihydro compounds are then re-oxidized to the corresponding riboflavin coenzymes by a number of specific

TABLE I

Enzyme	Source	Prosthetic group	Hydrogen donor	Hydrogen acceptor
Old yellow enzyme	Bottom yeast	Mononucleotide	Dihydro coenzymes I and II	Molecular oxygen
Cytochrome c reductase	Top yeast	Mononucleotide	Dihydro coenzyme II	Cytochrome c
Diaphorase I	Heart	Dinucleotide	Dihydro-coenzyme I	Cytochrome (a and b)
Diaphorase II	Bottom yeast	Dinucleotide	Dihydro-coenzyme II	?
Diaphorase	Milk	Dinucleotide	Dihydro-coenzyme I (and II?)	?
Aldehyde oxidase			Aldehyde	?
Xanthine oxidase			Xanthine	?
Aldehyde oxidase			Aldehydes	?
α -Amino-acid oxidase	Liver	Dinucleotide	α -Amino-acids	?
Glucose oxidase	Yeast	?	Glucose	?
?	Bottom yeast	Dinucleotide	?	Fumaric acid
Diamine oxidase	Kidney	?	D and polyamines	?
?	Top yeast	Dinucleotide	?	?

* D. R. Green, W. F. Knox and P. A. Stumpf, *J. Biol. Chem.* 138: 775 (1941)

reactions. Thus, the hydrogen may be transferred to fumaric acid or may react directly with oxygen or indirectly through the cytochromes *a*, *b* or *c*. The different reactions which are catalyzed by the riboflavin containing coenzymes and which have been elucidated are summarized in Table I.

Some of the enzymes listed in Table I apparently have a second prosthetic group in addition to the riboflavin coenzyme. This other compound is characterized by a brownish color but has not been identified. It has been observed in the flavin enzymes isolated from milk (diaphorase, aldehyde and xanthine oxidase), from liver (aldehyde oxidase) and from top yeast (action not characterized). Whether or not the group with the brownish color is the same in these three flavin enzymes has not been established.

The reactions catalyzed by the flavin enzymes as indicated in Table I are discussed more fully below.

Oxidation of the Codehydrogenases I and II This reaction can be accomplished by enzymes containing either the riboflavin mono or dinucleotide in the presence of specific apoenzymes. The mononucleotide containing flavin enzyme is the 'old' yellow enzyme of Warburg and Christian⁶². It is purified by adsorption methods^{63, 64} or by cataphoresis,⁶⁵ has a molecular weight of about 70,000⁶⁶ and a redox potential of -0.06 volts at pH 7.0 and 20° C. It is split into the apoenzyme and the coenzyme by denaturation of the apoenzyme with methanol or by dialysis against dilute hydrochloric acid. A resynthesis of the enzyme from the apoenzyme and the coenzyme can be accomplished. Riboflavin, if added to the apoenzyme in large excess, forms an active flavin enzyme⁶⁷ although the union is not quite as firm as with the mononucleotide. The riboflavin adenine dinucleotide is also able to combine with the specific apoenzyme and to react like the 'old' yellow enzyme. The chemistry of the apoenzyme is largely unknown but by hydrolysis the following amino acids have been obtained in a total yield of 65%: arginine, histidine, lysine, proline, tyrosine, phenyl alanine, tryptophane, cystine and glutamic acid.⁶⁸ The 'old' yellow enzyme dehydrogenates codehydrogenases I and II and

⁶² O. Warburg and W. Christian *Naturwissenschaften* 20: 688-980 (1932); *Biochem. Z.* 254: 438 (1932); 257: 492 (1933); 266: 377 (1933).

⁶³ F. Weygand and H. Stocker *Z. physiol. Chem.* 247: 167 (1937).

⁶⁴ F. Weygand and L. Birkofer *Ibid.* 261: 172 (1939).

⁶⁵ H. Theorell *Biochem. Z.* 275: 37-344 (1934); 278: 963 (1935).

⁶⁶ H. Theorell *Ibid.* 278: 279 (1935); R. A. Kekwick and K. O. Iredale *Biochem. J.* 30: 270 (1936).

⁶⁷ R. Kuhn and H. Rudy *Ber.* 69: 2557 (1936).

⁶⁸ R. Kuhn and P. Desnuelle *Ibid.* 70: 1907 (1937).

the dihydro form is dehydrogenated by oxygen. The rate of the last reaction is a function of the partial pressure of the oxygen present. In animal tissues the oxygen tension is so low that the oxidation can hardly be accomplished. Thus this reaction of the reduced riboflavin coenzyme with oxygen demonstrates a chemical function of the molecule but not its real physiological action. The reaction product of oxygen with the reduced riboflavin coenzyme is hydrogen peroxide which destroys the life of the cells. The latter has been demonstrated in the case of anaerobic growing lactic acid bacteria. Thus, if the old yellow enzyme plays an essential function in the living organism an oxygen transporting system must take care of the dehydrogenation of the dihydro riboflavin enzyme. It has been observed that cytochrome *c* reacts with the reduced 'old yellow' enzyme, but this reaction is too slow to be considered of physiological importance.^{69, 70} Fumaric acid^{71, 72} and glyoxal⁷³ have been suggested as substances capable of oxidizing the dihydro riboflavin system but convincing experimental evidences to substantiate these conceptions have not been reported.

An enzyme containing the riboflavin mononucleotide but an apoenzyme different from that of the old yellow enzyme has been found in the cytochrome *c* oxidase. This enzyme catalyzes the dehydrogenation of the coenzyme hydrogenase II and the reduction of the oxidized form of cytochrome *c*.⁷⁴ under physiological conditions.

Besides the enzyme systems which contain riboflavin mononucleotide and react with the dehydrogenases there are enzyme systems which contain the dinucleotide and which are capable of carrying out the same reactions. They are called the diaphorases,³ coenzyme factor⁷⁵ or pyridine nucleotide oxidase and occur in animal and in plant tissues.⁷⁷ The enzymes from heart, skeletal muscle and yeast are apparently identical.^{78, 79, 80} The same enzyme or enzymes also occur in bacteria.⁸¹ There are two dif-

⁶⁹ H. Theorell, *Angew. Chem.* 51, 738 (1938).

⁷⁰ H. Theorell, *Nature* 138, 687 (1936); *Biochem. Z.* 288, 31 (1936); 279, 463 (1935).

⁷¹ E. Adler and H. v. Euler, *Akt. Kems. Sk. anal. Grol. Biol.* No. 38 (1937).

⁷² A. Szent-Györgyi, *Z. physiol. Chem.* 244, 105 (1936).

⁷³ T. Bersin, *S. B. Ges. Bef. ges. Naturw. Ma. burg* 71, 56 (1936).

⁷⁴ P. Haas, B. L. Horeck and T. R. Hogness, *J. Biol. Chem.* 136, 747 (1940).

⁷⁵ H. v. Euler and H. Hellström, *Z. physiol. Chem.* 252, 31 (1939); H. v. Euler and K. Hasse, *Naturwissenschaften* 26, 187 (1938); H. v. Euler and G. Günther, *Ibid.* 26, 676 (1938).

⁷⁶ J. G. Dewan and D. F. Green, *Biochem. J.* 32, 6 (1938); P. B. Straub, H. S. Corran and D. F. Green, *Nature* 143, 76, 119 (1939).

⁷⁷ E. B. Lockhart, *Biochem. J.* 33, 613 (1939).

⁷⁸ P. B. Straub, *Ibid.* 33, 787 (1939).

⁷⁹ H. S. Corran, D. F. Green and P. B. Straub, *Ibid.* 33, 793 (1939).

⁸⁰ P. Haas, *Biochem. Z.* 298, 378 (1938).

⁸¹ D. F. Green and J. G. Dewan, *Biochem. J.* 32, 676 (1938).

ferent diaphorases, namely, diaphorase I which dehydrogenates codehydrogenase I, and diaphorase II, which dehydrogenates codehydrogenase II.⁸² The reversed reaction, the dehydrogenation of the reduced diaphorases, is probably carried out by cytochromes *a* and *b*. It has been shown experimentally that diaphorase I reacts with cytochrome *b*⁸³ and some evidence has been obtained for the participation of cytochromes *a* and *b* in the dehydrogenation of the diaphorases.⁸⁴

The apoenzyme of the diaphorase I has a molecular weight of about 70,000. It appears that the combination of the apoenzyme with the coenzyme is rather loose and that the coenzyme may change from one apoenzyme molecule to another with considerable speed. On the other hand, the same specific protein may possibly serve as apoenzyme for both the coenzymes of the riboflavin and the nicotinamide type.⁸⁵ One mol of the diaphorase catalyzes the oxidation of about 8000 mols of codehydrogenase I under optimum conditions in a synthetic system containing methylene blue as an oxygen carrier.⁸⁶

Through the codehydrogenases the riboflavin enzyme systems take part⁸⁷ in the reactions carried out by the nicotinamide containing enzymes⁸⁸ (see page 227), such as in the dehydrogenation of hexose monophosphate in yeast⁸⁹ and muscle,⁹⁰ of alcohol in yeast,⁹¹ of glucose in liver,⁹² of malic acid in muscle,⁹³ of lactic acid in muscle,⁹⁴ of citric acid in muscle and in plant cells,⁹⁵ of dioxo acetone phosphate in yeast,⁹⁶ of glycerin aldehyde phosphate in yeast⁹⁷ and of glycerin phosphoric acid in seeds.⁹⁸

⁸² E. Adler, H. v. Euler, G. Günther and E. D. Plass, *Skand. Arch. Physiol.* **82**, 61 (1939); E. Adler, H. v. Euler and G. Günther, *Nature* **143**, 641 (1939); E. P. Abraham and E. Adler, *Biochem. J.* **34**, 119 (1940).

⁸³ K. Okunuki and E. Yakusiji, *Proc. Imp. Acad. (Tokyo)* **16**, 144 (1940).

⁸⁴ J. G. Dewan and D. E. Green, *Biochem. J.* **32**, 626 (1938).

⁸⁵ E. Haas, *Biochem. Z.* **290**, 291 (1937).

⁸⁶ E. Haas, *Ibid.* **298**, 378 (1938); H. S. Corran, D. E. Green and F. B. Straub, *Biochem. J.* **33**, 793 (1939).

⁸⁷ H. Theorell, *Ergeb. Enzymforsch.* **6**, 132 (1937); O. Warburg, *Ibid.* **7**, 210 (1938).

⁸⁸ O. Warburg, W. Christian and H. Griese, *Biochem. Z.* **282**, 167 (1935).

⁸⁹ O. Warburg and W. Christian, *Ibid.* **254**, 438 (1932); **257**, 492 (1933); **266**, 377 (1933).

⁹⁰ T. Wagner Jauregg, *Z. physiol. Chem.* **231**, 55 (1935).

⁹¹ H. v. Euler and E. Adler, *Ibid.* **226**, 195 (1934).

⁹² E. Adler and H. v. Euler, *Ibid.* **232**, 6 (1935).

⁹³ T. Wagner Jauregg, *Ibid.* **228**, 273 (1934); **231**, 55 (1935); E. Adler and M. Michaelis, *Ibid.* **238**, 261 (1935).

⁹⁴ E. Adler and M. Michaelis, *Ibid.* **235**, 154 (1935); **238**, 261 (1936); T. Wagner Jauregg and E. F. Möller, *Ibid.* **236**, 216 (1935).

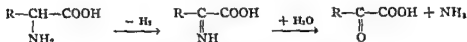
⁹⁵ T. Wagner Jauregg, *Ibid.* **233**, 215 (1935).

⁹⁶ H. v. Euler, E. Adler and H. Hellström, *Ibid.* **241**, 239 (1936).

⁹⁷ H. v. Euler, E. Adler and H. Hellström, *Ibid.* **241**, 239 (1936).

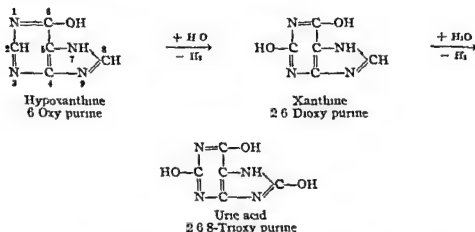
⁹⁸ T. Wagner Jauregg and H. Rauem, *Ibid.* **237**, 233 (1935).

Oxidation of *d*-Amino-acids The oxidation of the antipodes of the naturally occurring amino acids into oxo acids is carried out by an enzyme system containing riboflavin adenine dinucleotide⁹⁹⁻¹⁰¹ according to the following reaction scheme¹⁰²



One mol of the riboflavin adenine dinucleotide transports in the presence of an excess of the apoenzyme approximately 2000 mols of oxygen per minute at 38° C. The apoenzyme has a molecular weight of about 65 000. The coenzyme is highly dissociated from the apoenzyme in solution but the reduced coenzyme apparently is not dissociated.

Oxidation of Xanthine By means of an enzyme system containing riboflavin adenine dinucleotide plus an unknown colored compound several purines, primarily xanthine and hypoxanthine, are dehydrogenated to uric acid¹⁰³⁻¹⁰⁶



The conversion of hypoxanthine to xanthine and the conversion of xanthine to uric acid require the presence of the same riboflavin enzyme. One mol

⁹⁹ O. Warburg and W. Christian *Biochem. Z.* 295: 261 (1938); 298: 150 (1938).

¹⁰⁰ E. Neglein and H. Brömmel *Ibid.* 300: 275 (1939).

¹⁰¹ F. B. Straub *Nature* 141: 603 (1938).

¹⁰² H. A. Krebs *Z. physiol. Chem.* 217: 191 (1933); 218: 157 (1933); *Biochem. J.* 29: 1620 (1935).

¹⁰³ E. G. Ball *Science* 88: 131 (1938); *Angew. Chem.* 51: 738 (1938); *J. Biol. Chem.* 128: 51 (1939).

¹⁰⁴ H. S. Corran and D. E. Green *Biochem. J.* 32: 2231 (1938).

¹⁰⁵ D. E. Green and H. S. Corran *Angew. Chem.* 51: 739 (1938).

¹⁰⁶ H. S. Corran, J. G. Dewar, A. H. Gordon and D. E. Green *Biochem. J.* 34: 1694 (1939).

of the coenzyme catalyzes the oxidation of about 300 mols of hypoxanthine or xanthine per minute. The oxidation rate of other purines is considerably slower.¹⁰⁷

The same enzyme system is also able to effect an anaerobic dismutation of xanthine to hypoxanthine and uric acid



This is an equilibrium reaction and xanthine can therefore be formed from uric acid and hypoxanthine.^{108, 109}

This riboflavin containing enzyme xanthine oxidase, occurs for example, in milk and liver. The apoenzyme has a molecular weight of about 280,000¹¹⁰ and probably binds two riboflavin groups per molecule in addition to some other unknown colored group which may act as coenzyme. The apoenzyme is different from those of the other riboflavin containing enzyme systems.

Oxidation of Aldehydes The enzyme which catalyzes the oxidation of aldehydes to carboxylic acids, for example, propionic aldehyde to propionic acid, was originally observed in milk and is called aldehyde oxidase or Schardinger enzyme.¹¹¹ The aldehyde oxidase from liver^{112, 113} contains the riboflavin adenine dinucleotide and is specific for the oxidation of aldehydes. The aldehyde oxidase from milk also acts as xanthine oxidase and as dihydro codehydrogenase I oxidase. Whether or not these enzymes are identical has not been decided. There is also a discrepancy of opinion as to whether or not the aldehyde oxidase and xanthine oxidase from liver are a single compound or a mixture of compounds. Much evidence has accumulated which favors the view that a single compound acts as an enzyme in milk for the oxidation of both xanthine and aldehydes.^{114, 115} If that is the case, the aldehyde oxidase from liver must be a different compound.

Oxidation of Diamines A riboflavin containing coenzyme of unknown composition takes part in the enzymatic conversion of di- and poly-

¹⁰⁷ M. Dixon *Enzymologia* 5: 198 (1938). L. Reichel and W. Burkart *Z. physiol. Chem.* 260: 135 (1939).

¹⁰⁸ D. E. Green *Biochem. J.* 28: 1550 (1934).

¹⁰⁹ S. Filippini *J. chim. phys.* 32: 1 (1935).

¹¹⁰ J. S. L. Philpot *Biochem. J.* 32: 2240 (1938).

¹¹¹ F. Schardinger *Z. Untersuch. Nahr. u. Genussm.* 5: 1113 (1902).

¹¹² V. Subrahmanyam, D. E. Green and A. H. Gordon *Nature* 144: 1016 (1939).

¹¹³ A. H. Gordon, D. E. Green and V. Subrahmanyam *Biochem. J.* 34: 764 (1940).

¹¹⁴ V. H. Booth *Ibid.* 32: 494 (1938).

¹¹⁵ M. Dixon *Enzymologia* 5: 198 (1938). I. Reichel and W. Burkart *Z. physiol. Chem.* 260: 135 (1939).

amines into amino aldehydes¹¹⁶ This action is similar to the oxidation of α amino acids, but the enzymes (at least the apoenzymes) involved appear to be different The amines studied in this reaction are primarily histamine, cadaverin and spermin

Oxidation of Glucose The oxidation of glucose to gluconic acid in yeast is carried out with the aid of an enzyme which contains riboflavin¹¹⁷⁻¹¹⁹ The manner in which riboflavin is bound in this enzyme system is not known

Reduction of Fumaric Acid In yeast fumaric acid is reduced to succinic acid by an enzyme system which contains riboflavin adenine dinucleotide¹¹⁹ Per mol of dinucleotide 2000-3000 mols of hydrogen are transported per minute This enzyme is also able to reduce other compounds such as maleic acid crotyl alcohol phenyl crotyl alcohol and geraniol but the rate of reduction is less than $1/10$ that of the rate of fumarate reduction The specificity of the apoenzyme has not been determined but it appears probable that the apoenzyme of at least one of the oxidases previously discussed is identical with that of the fumaric hydrogenase

Reduction of Cytochrome a , b and c The reduction of the oxidized form of the cytochromes is linked with the oxidation of the dihydrocodehydrogenases (see page 174)

(b) Coenzymes Containing Riboflavin

Riboflavin-5'-phosphoric Acid (Riboflavin-mononucleotide) Riboflavin 5'-phosphoric acid was first obtained from heart muscle and was characterized as a yellow water soluble dye This compound was given the name 'cytostav'¹²⁰ The position of the phosphoric acid in the riboflavin molecule was established by oxidation with periodic acid¹²¹ Formaldehyde was not obtained as a reaction product as would be expected in case a free primary hydroxyl group should be present in the 5' position This result was confirmed by synthesis which was carried out according to the following scheme¹²

¹¹⁶ P. A. Zeller, R. Stern and J. N. Wenk, *Helv. Chim. Acta* 23, 1 (1940)

W. Franke and M. D. D. H. 541, 117 (1939)

¹¹⁷ D. Mueller, *Biochem. Z.* 199, 136 (1926); 205, 111 (1929); 213, 211 (1929); 232, 473 (1931)

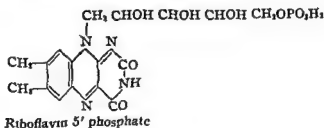
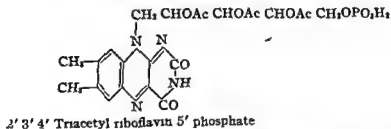
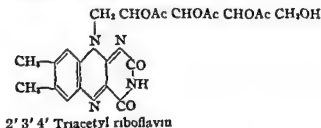
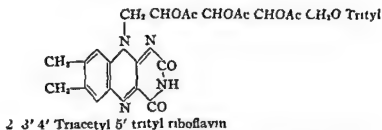
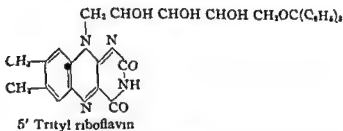
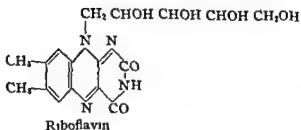
W. Franke and M. D. D. H. 532, 1 (1937)

¹¹⁸ F. G. Fisher, A. Roedger and K. Rauch, *Naturwissenschaften* 27, 197 (1939); F. G. Fisher and H. Fytenbach, *Ann.* 529, 87 (1937); 530, 99 (1937)

¹¹⁹ I. Banga and A. Szent-Györgyi, *Biochem. Z.* 246, 703 (1931)

¹²⁰ P. Karrer, P. Ires and H. Meerwein, *Helv. Chim. Acta* 20, 79 (1937)

¹²¹ R. Kuhn, H. R. dy and F. W. yland, *Ber.* 69, 1543 (1936); R. Kuhn and H. R. dy, *Ibid.* 69, 1974 (1936)



The phosphorylation of riboflavin *in vitro* has also been carried out by a phosphatase concentrate prepared as a dry powder from the intestinal mucosa of rats, cats and pigs,¹¹³ and by a glycerol extract of the small intestine of rats in 0.01 molar phosphate solution.¹¹⁴

The properties of riboflavin 5' phosphoric acid correspond closely to those of the free riboflavin. The ester is considerably more soluble in water than the free riboflavin, and can be precipitated in the form of various salts. Lumiflavin and lumichrome, respectively, are formed upon irradiation similar to the formation of these compounds from riboflavin. In cataphoresis experiments riboflavin does not move; the phosphoric acid ester, however, migrates to the anode.

In neutral solution, the ester is quite stable. Hydrolysis occurs rapidly in acid solution, but considerably more slowly in alkaline medium. The ester is also hydrolyzed by phosphatases such as the α glycerol phosphatase.

Riboflavin 5' phosphoric acid combines with specific proteins, the apoenzymes, by attachment at two points, namely, at the phosphoric acid group and at the slightly acidic imino group in 3 position of the riboflavin molecule. In accordance with this conception, flavins which are substituted in the 3 position do not form enzyme systems¹¹⁵ and are also devoid of vitamin activity. Furthermore, the typical fluorescence of riboflavin is dependent upon the presence of a free β imino group and neither 3 substituted riboflavins nor the enzyme systems exhibit fluorescence.

Riboflavin-adenine-dinucleotide The riboflavin adenine dinucleotide has the composition $C_{27}H_{47}O_{13}N_9P_2$ and forms salts such as a monobarium and monosodium salt. The constitution of the dinucleotide has not been well defined. By enzymatic¹¹⁶ or by acid¹¹⁷ hydrolysis the dinucleotide is split into two mononucleotides, namely, into riboflavin 5'-phosphoric acid and adenylic acid (adenosine 5' monophosphoric acid). (See formula on following page.)

The dinucleotide is widely distributed in animal tissues and in microorganisms. It is also believed to occur in plants. It has been isolated from liver, kidney, heart, muscles, Jensen sarcoma (a special type of rat tumor) and yeast.^{118, 119} The isolation involves a denaturation of the apoenzyme.

¹¹³ H. Hübner and F. Verár, *Helv. Chim. Acta* 21, 1006 (1938); R. Pulver and F. Verár, *Enzymologia* 6, 333 (1939).

¹¹⁴ H. Rudy, *Naturwissenschaften* 23, 296 (1936).

¹¹⁵ R. Kuhn and H. Rudy, *Ber.* 69, 757 (1936).

¹¹⁶ O. Warburg and W. Christian, *Biochem. Z.* 298, 150, 368 (1938).

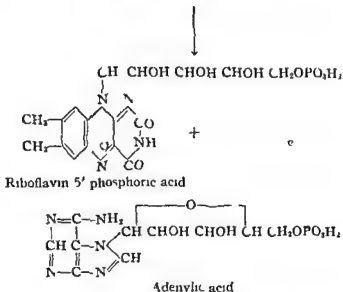
¹¹⁷ L. P. Abraham, *Biochem. J.* 33, 543 (1939).

¹¹⁸ P. Karrer, P. Frei and M. Meerwein, *Helv. Chim. Acta* 20, 79 (1937); P. Karrer, P. Frei and H. Kungler, and H. Benda, *Ibid.* 21, 86 (1938).

¹¹⁹ O. Warburg, W. Christian and A. C. Reese, *Biochem. Z.* 295, 41 (1938); 297, 417 (1938); 298, 150 (1938).

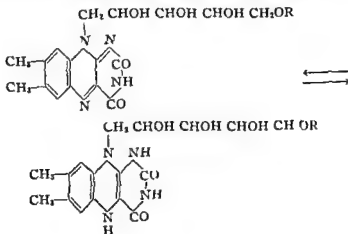
zyme by heat treatment (75° C) followed by extraction with phenol. From the phenol solution the dinucleotide is extracted with water in the presence of ether. From the acidified water solution the dinucleotide is precipitated as the silver salt and after conversion into the barium salt the latter is recrystallized from water.

Riboflavin adenine dinucleotide



(c) Mechanism of the Coenzyme Action

Riboflavin acts in various enzyme systems by reversibly accepting and donating two atoms of hydrogen. This is accomplished by the addition of hydrogen to the 1 and 10 positions of riboflavin, thus forming the previously discussed dihydro or leuco riboflavin (see page 160).



10 Specificity

Riboflavin is the only naturally occurring flavin with vitamin B₂ activity. A great number of flavin compounds have been prepared synthetically and have been tested for vitamin activity. Besides riboflavin the following compounds were found to exhibit vitamin B₂ activity:

- 7 Methyl 9 (*d*,1' ribityl) isoalloxazine¹³⁰
- 6 Methyl 9 (*d*,1' ribityl) isoalloxazine¹³⁰
- 6 Ethyl 7 methyl 9 (*d*,1' ribityl) isoalloxazine¹³¹

Whereas rats need about 0.7 of riboflavin per day they require about twice as much of these three compounds. Similar results are obtained when lactic acid bacteria are used for the evaluation of the vitamin activity.¹³²

The following compounds possess some stimulating activity for rats and lactic acid bacteria in the presence of suboptimal amounts of riboflavin:

- 6,7 Dimethyl 9 (*l*,1' arabityl) isoalloxazine¹³³
- 6,7 Dimethyl 9 (*d*,1' arabityl) isoalloxazine¹³⁴ ¹³⁵
- 7 Ethyl 9 (*d*,1' ribityl) isoalloxazine¹³⁶
- 6,7 Trimethylene 9 (*l*,1' arabityl) isoalloxazine¹³⁷
- 6,7 Tetramethylene 9 (*l*,1' arabityl) isoalloxazine¹³⁷

Riboflavin tetra acetate and diacetone riboflavin are active on rats, probably due to hydrolysis in the organism. The tetra acetate is, however, inactive on lactic acid bacteria. The 3 phosphoric acid ester of riboflavin¹³⁸ and the flavin adenine dinucleotide show full vitamin activity. The flavin glucosides are inactive.

Generally speaking substitution in 6 or 7 position is necessary for the vitamin activity. Absence of substituents in both positions is accompanied by high toxicity¹³⁹. An unsubstituted imino group in 3 position is also necessary. Riboflavins which are alkylated in 3 position are totally devoid of vitamin and coenzyme activity.

¹³⁰ P. Karrer, H. v. Euler, M. Malmberg and K. Schöpp, *Helv. Chim. Acta* 7, 147, 153 (1935). P. Karrer, H. Salomon, K. Schöpp, I. Benz and B. Beck, *Helv. Chim. Acta* 18, 908 (1935).

¹³¹ P. Karrer and T. H. Quibell, *Helv. Chim. Acta* 19, 1034 (1936).

¹³² P. Karrer, H. Salomon, K. Schöpp, I. Schlatter and H. Fritzsche, *Ibid.* 17, 1010 (1934).

¹³³ H. v. Euler, P. Karrer, M. Malmberg, K. Schöpp, F. Benz, B. Becker and P. Frei, *Ibid.* 18, 52 (1935). P. Karrer, H. v. Euler, M. Malmberg, K. Schöpp and P. Benz, *Svensk. Kemi. Tidn.* 47, 93 (1935). P. György, *Z. Vitaminforsch.* 4, 3 (1935).

¹³⁴ H. v. Euler, P. Karrer and M. Malmberg, *Helv. Chim. Acta* 18, 1376 (1935).

¹³⁵ Vitamin activity questionable.

¹³⁶ P. Karrer and T. H. Quibell, *Helv. Chim. Acta* 19, 1034 (1936).

¹³⁷ R. Kuhn, H. Vetter and H. W. Rzepp, *Ber.* 70, 1307 (1937).

¹³⁸ P. György, *Proc. Soc. Exptl. Biol. Med.* 35, 707 (1936).

¹³⁹ R. Kuhn and P. Boulanger, *Z. physiol. Chem.* 241, 233 (1936).

11 Determination

(a) Physical Methods

1 Determination of the Absorption Spectrum The determination of vitamin B₂ by its characteristic ultraviolet absorption spectrum is suitable only for solutions of the pure compound, and this method has the additional disadvantage that the vitamin is sensitive to light. Thus during the determination the vitamin is at least partly destroyed, and the material destroyed before the beginning of the determination adds to the apparent value of the vitamin.

2 Determination of the Fluorescence Spectrum Vitamin B₂ can be determined by its characteristic fluorescence spectrum¹⁴⁰ which shows a maximum at 565 mμ at pH 6. It is best to compare the intensity of the fluorescence with some standard such as pure riboflavin,¹⁴¹ potassium dichromate,¹⁴² fluorescein¹⁴³ or uranium glass.¹⁴⁴ The fluorescence is directly proportional to the vitamin content. Relatively reliable results have been obtained with this method when the vitamin B₂ content of animal tissues or milk is assayed. The method becomes inaccurate, however, in cases when other fluorescent substances are present as in the case of urine and yeast extracts. Another source of error often encountered is a non quantitative extraction of the vitamin. Such extractions are often necessary to separate riboflavin from interfering substances. A method which has given results in agreement with biological assays consists in enzymatic hydrolysis of, for example, tissues, followed by extraction with water and determination of the fluorescence of the extract.¹⁴⁵

Several modifications of this basic assay procedure have been recommended. Thus, vitamin B₂ has been determined indirectly by the fluorescence of the vitamin before and after reduction with sodium hydrosulfite.¹⁴⁶ This has been possible since the reduced vitamin does not show the typical fluorescence of the unreduced vitamin. Another modification is to determine, first, the total fluorescence of a given solution and, second, the fluorescence of the interfering substances by destroying the vitamin through alkali.¹⁴⁷

¹⁴⁰ H. v. Euler and E. Adler, *Svensk Kem. Tid.* **45**, 276 (1933); *Z. physiol. Chem.* **223**, 105 (1934).
 G. C. Supplee, S. Ansbacher, G. E. Flanagan and Z. M. Hanford, *J. Dairy Sci.* **19**, 215 (1936).

¹⁴¹ A. Z. Hodson and I. C. Norris, *J. Biol. Chem.* **131**, 621 (1939).

¹⁴² A. J. Chante and N. W. Khaustov, *Biochem. J.* **29**, 34 (1935). G. N. Murthy, *Indian J. Med. Research* **24**, 1083 (1937).

¹⁴³ F. H. Cohen, *Rec. trav. chim.* **54**, 133 (1935). S. M. Weisberg and I. Le, in *Ind. Eng. Chem. Anal. Ed.* **9**, 523 (1937).

¹⁴⁴ D. B. Hand, *Ind. Eng. Chem. Anal. Ed.* **11**, 306 (1939).

¹⁴⁵ P. O. Van Duvne, *J. Biol. Chem.* **139**, 707 (1941).

¹⁴⁶ A. Z. Hodson and I. C. Norris, *Ibid.* **131**, 671 (1939).

¹⁴⁷ H. Kahler and E. P. Davis, *Proc. Soc. Exptl. Biol. Med.* **44**, 104 (1940).

*3 Polarographic Determination It has been proposed to determine vitamin B₂ by the polarographic technique¹⁴⁸ Assays have been carried out so far only on relatively pure vitamin B₂ preparations and the method needs further study to determine whether or not this procedure can be applied for the determination of riboflavin in natural products

4 Lumi-Lactoflavin Method This method is based on the conversion of riboflavin into lumi lactoflavin by irradiation in alkaline solution¹⁴⁹ The so formed lumi lactoflavin is extracted from the acidified solution with chloroform and determined by its absorption in the fluorescence spectrometer¹⁴⁹ The accuracy of this method is limited, since the conversion of riboflavin into lumi lactoflavin is by no means quantitative Nevertheless this method proved to be effective if the conversion rate of pure riboflavin in a solution of about the same concentration as in the solution to be examined is determined separately taking care that the same pH, temperature and amount of air are present In another experiment a known amount of pure riboflavin is added to the unknown solution and the conversion is determined By proper correlation of the three figures obtained an accurate determination of the vitamin B₂ content of almost any solution can be achieved

The separation of riboflavin from its phosphoric acid ester is carried out by extraction of the aqueous solution with benzyl alcohol, in which only the free riboflavin is soluble¹⁵⁰

The separation of riboflavin and riboflavin 5' phosphoric acid from flavin combined with proteins is achieved by dialysis in cellophane tubes at 3° C for 16 hours

(b) Biological Methods

1 Rat Growth Test The most accurate and generally accepted biological method for the determination of riboflavin is the curative growth test on rats^{151 152 153} Young rats kept on a riboflavin free diet stop

¹⁴⁸ J J Lingane and O L Daniels *J Biol Chem* 137 567 (1941)

¹⁴⁹ R Kubo, F Wagner Jauregg and H Kaltschmitt *Ber* 67 1452 (1934) H v Euler E Adler and A Schlötter *Z physik Chem* 226 87 (1934) P Vivanco *Naturwissenschaften* 23 306 (1935)

¹⁵⁰ A Emmene Ascis 141 416 (1938) *Rec trav chim* 58 790 (1939)

¹⁵¹ A Bourquin and H C Sherman *J Am Chem Soc* 53 3501 (1931)

¹⁵² P György I W van Klaveren R Kubo and T Wagner Jauregg *Z physiol Chem* 223 236 (1934)

¹⁵³ A Bourquin and H C Sherman *J Am Chem Soc* 53 3501 (1931) H E Munell *J Nutrition* 4 203 (1931) P György *Biochem J* 29 741 (1935) P L Dyand W O Langton *J Nutrition* 7 17 (1934) G C Supplee R C Bender and O G Jen en *Ind Eng Chem Anal Ed* 11 495 (1939) T s Hamilton and H H Mitch II *J Nutrition* 10 117 (1935) L A Randon A Raffy and J Aqueres *La Compl end soc b* 126 877 (1937) H Lindholm *Biochem J* 32 314 (1938) E V Carlsson and H C Sherman *J Nutrition* 13 57 (1938) M M Fl Sadr T P Macrae and C E Work *Biochem J* 34 601 (1940) J R Wagner A F Axelrod M A Lipton and C A Fivchjem *J Biol Chem* 136 337 (1940)

growing within a few days. Addition of riboflavin to the diet causes continuation of growth. The same principle has also been used in a prophylactic method.

2 Chick Growth Test An apparently very reliable method is the growth response of chicks, which is within certain limits a linear function of the amount of riboflavin administered.¹⁵⁴

3 Lactic Acid Bacteria Test This method¹⁵⁵ is based on the essential nature of riboflavin for the growth of *Lactobacillus casei*. The evaluation is carried out by measurement of the turbidity produced by the growth of the organism or by titration of the lactic acid formed. This method determines not only riboflavin in the free form, but also in the combined forms (coenzymes and enzymes).^{156 157}

(c) Biochemical Methods

1 Yellow Enzyme Test¹⁵⁸ The oxygen uptake of the following system is measured: Hexose-phosphoric acid, codehydrogenase II (page 235), apodehydrogenase and the specific protein of the yellow enzyme. The rate of the oxygen uptake per minute is a function of the amount of the riboflavin added to this system.

2 Determination of Riboflavin-adenine-dinucleotide¹⁵⁹ The dinucleotide is determined by its ability to act in combination with a specific protein as *d* amino acid oxidase.^{160 161 162} Actually, the rate of oxygen uptake is measured during the oxidation and is compared with the rate of oxygen uptake catalyzed by known amounts of the dinucleotide in the same system.

12 Standards

One B. S. Rat Unit (Bourquin Sherman Unit) is defined as the daily amount of riboflavin required by rats to insure proper growth (10 g increase of body weight per week for two to four weeks).

¹⁵⁴ T. H. Jukes, *J. Nutrition* **14**, 223 (1937).

¹⁵⁵ E. E. Snell and F. M. Strong, *Ind. Eng. Chem. Anal. Ed.* **11**, 346 (1939); *Enzymologia* **6**, 186 (1939); E. E. Snell, F. M. Strong and W. H. Peterson, *Biochem. J.* **31**, 1789 (1937); *J. Am. Chem. Soc.* **60**, 2875 (1938).

¹⁵⁶ R. E. Feeney and F. M. Strong, *Proc. Am. Soc. Biol. Chem.* **1940**, XXXI.

¹⁵⁷ F. M. Strong, R. E. Feeney, B. Moore and H. T. Parsons, *J. Biol. Chem.* **137**, 363 (1941).

¹⁵⁸ R. Kuhn and H. Rudy, *Ber.* **69**, 257 (1936).

¹⁵⁹ O. Warburg and W. Christian, *Biochem. Z.* **298**, 160, 368 (1938).

¹⁶⁰ E. Negelein and H. Brömel, *Ibid.* **300**, 225 (1939).

¹⁶¹ J. R. Klein and H. I. Kohu, *J. Biol. Chem.* **136**, 177 (1940).

¹⁶² S. Ochoa and R. J. Rosier, *Biochem. J.* **33**, 008 (1939).

An International Unit of riboflavin has not been established. Von Euler¹⁶³ proposed as International Unit 5 γ of pure crystallized riboflavin, which amount produces an increase in weight of 0.8–1.0 g per day in young rats.

1 Cornell Unit of riboflavin = 1 γ of riboflavin defined by the growth effect on chicks¹⁶⁴

1 B S Unit = 20–30 γ of riboflavin^{165, 166}

1 g riboflavin = 400 000–500 000 B S Units

13 Physiology of Plants and Microorganisms

The physiology of plants and microorganisms with respect to the action of riboflavin in their cells has not been studied sufficiently. It is however reasonable to assume that principally the same reactions are carried out by riboflavin in plants and in microorganisms as in animals, especially as part of enzyme systems.

Most microorganisms, bacteria¹⁶⁷, molds¹⁶⁸, fungi¹⁶⁹ etc., synthesize riboflavin, just as the higher plants do. *Microbaccillus tuberculosis*, for example, produces 0.5 to 2.9 γ of riboflavin per day¹⁷⁰. A few, however, have been found, for example special strains of lactic acid bacteria¹⁷¹ and of the streptococci¹⁷² which require an outside supply of riboflavin and are thus parasitic in character (see page 193).

In anaerobic cells, for example in yeast, in lactic and butyric acid bacteria, the riboflavin containing enzyme systems maintain the respiration¹⁷³ or that part of the respiration which cannot be disturbed by either hydrocyanic acid or by carbon monoxide and which therefore is not a function of a porphyrin enzyme system which contains iron and is easily poisoned. Aerobic living bacteria contain and apparently need considerably less riboflavin than do the anaerobic living organisms.

Riboflavin is, as far as is known, synthesized by all higher plants. It is apparently formed in the green leaves where it is found predomi-

¹⁶³ H. v. Euler: Institut international de Chimie Solvay. 5^eème Conseil de Chimie: rapport et discussions. *Les Vitamines et l'Homme*. Paris 1938, p. 199.

¹⁶⁴ L. C. Norris, H. S. Wilgus, A. T. Ringrose, V. H. M. N. A. C. 1. He. Ser. *Cornell Univ. Agr. Expt. Sta. Bull.* 660, 3 (1936).

¹⁶⁵ O. A. Bessey: *J. Nutrition* 15, 11 (1938).

¹⁶⁶ H. v. Euler, P. Karrer, F. Adler and M. M. Imberg: *Helv. Chim. Acta* 17, 11, 7 (1934).

¹⁶⁷ O. W. Rburg and W. Ch. H. in: *Biochem. Z.* 266, 377 (1933).

¹⁶⁸ J. L. Olay and F. Laborey: *Compt. rend.* 204, 1696 (1937); 205, 170 (1937); 206, 305 (1938).

¹⁶⁹ A. C. H. rmo d. M. Fontaine and A. R. R. y. *ibid.* 201, 1077 (1935); A. Raffy: *ibid.* 209, 900 (1935).

¹⁷⁰ C. H. Rousseau, W. F. Dren and H. W. Schultz: *Proc. Soc. Exptl. Biol. Med.* 39, 481 (1935).

¹⁷¹ F. E. Snell and F. M. Stru. *g. Enzymology* 6, 186 (1939).

¹⁷² F. J. Krauskopf, F. E. Snell and K. McCoy: *ibid.* 7, 377 (1939).

¹⁷³ M. Doudroff: *ibid.* 3, 279 (1938).

nantly In broccoli, the flower buds contain only a little over half as much as the leaves and the twigs contain even less ¹⁷⁴ In carrots the riboflavin content of the roots is only one fourth of the content of the tops It has been shown that excised cosmos roots need a supply of vitamin B₂ ¹⁷⁵ which apparently is furnished in the intact plant from some other part, probably the leaves A beneficial effect on the growth of eggplants in synthetic nutrient solution has been observed upon the addition of 2.5% of riboflavin ¹⁷⁶ There seems to exist a definite species difference in the response of various plants to riboflavin administration

As leaves become older, the riboflavin content diminishes Younger parts of plants always seem to contain more than older parts Ungerminated seeds generally contain little riboflavin (peas are an exception) During germination the riboflavin content increases many times

It has been reported that riboflavin increases the phototropic action of plants ¹⁷⁷

14 Animal Physiology

(a) General Physiology, Metabolism and Mechanism of the Vitamin B₂ Action

The chemically bound vitamin B₂, as it occurs, for example in vegetables and in seeds, cannot be absorbed in the intestines of animals as has been shown in the case of the rat ¹⁷⁸ After cooking, however, the vitamin is liberated and completely absorbed

The three naturally occurring free riboflavin compounds namely, riboflavin, riboflavin 5' phosphoric acid and riboflavin adenine dinucleotide are easily absorbed by the intestines in the small gut The free riboflavin is phosphorylated in the intestines as can be shown *in vitro* with dried powdered intestinal mucosa or with glycerol extracts of intestines When the phosphorylation mechanism is disturbed, for example, experimentally with iodoacetic acid or by extirpation of the adrenals ¹⁷⁹ riboflavin is not absorbed and the organism (rats were used for these experiments) ceases growing Only by the addition of riboflavin phosphoric acid (and possibly also of the dinucleotide) to the diet does further growth occur

¹⁷⁴ Mentioned in the review by H. C. Sherman and C. S. Lanford in *The Vitamins* Am. Med. Assoc. 1939, p. 292

¹⁷⁵ J. Bonner *Am. Chem. Soc. Div. Agr. Food Chem. Meeting* Sept. 1939, Abst. 13-14

¹⁷⁶ R. Dennison *Science* 92, 17 (1940)

¹⁷⁷ M. Heiman *Wien. klin. Wochschr.* 49, 398 (1936)

¹⁷⁸ F. M. Iantze *Agr. Exptl. Sta. New Mexico Coll. Agr. Mech. Arts Bull.* 268 (1939)

¹⁷⁹ J. Verzar and L. Jasst *Arch. ges. Physiol. (Pflügers)* 236, 693 (1935); 237, 476, 483 (1935); *Zeitschr. f. physiol. Biol.* 1, VI (1936); *Enzymologia* 3, 16 (1937)

The transformation of riboflavin to its phosphoric acid ester and the dinucleotide is also a general cellular reaction. Human blood cells, for example but not the plasma, can synthesize the dinucleotide from riboflavin both *in vitro* and *in vivo* ¹⁸⁰. Therefore, riboflavin can also be administered parenterally.

Riboflavin phosphoric acid is built into various enzyme systems which have been discussed previously. The liver and kidney seem to undertake these reactions to a greater extent than the other organs.

Riboflavin is excreted predominantly in the feces, and to a smaller extent in urine. During vitamin B₂ avitaminosis, no riboflavin is excreted in the urine; small amounts however are found in the feces. Vitamin B₂ is excreted mainly in the free form but in varying amounts, up to 50%, also as phosphoric acid ester. An increase in the riboflavin intake of humans increases the urinary output of riboflavin ^{180, 181}. The intake of riboflavin phosphoric acid increases to a small extent the excretion of the phosphorylated compound besides increasing to an appreciable extent the excretion of free riboflavin ¹⁸. Normal human beings on a balanced diet excrete about 500-800 γ per day ¹⁸³.

Besides riboflavin and its phosphoric acid ester another flavin called aquoflavin or uroflavin is found in urine. The chemical constitution of this compound is not quite clear; it seems to contain more oxygen than does riboflavin and is more water soluble. It is also sensitive to illumination and appears to be similarly converted into a lumiflavin compound ¹⁸⁴. Uroflavin is apparently a degradation product of riboflavin.

The animal organism has no special storage organs for riboflavin, although a certain level is maintained in the various tissues (0.5 γ per gram of blood ¹⁸⁵). The flavin dinucleotide level in blood cells and plasma is fairly constant ¹⁸⁰. Relatively large amounts are found for example in liver and in kidney. Intake of large amounts of riboflavin does not increase the riboflavin content of the liver to any appreciable extent ¹⁸⁶ but increases the dinucleotide concentration in the blood cells ¹⁸⁰. On the other hand, the organs of animals which die of vitamin B₂ avitaminosis still contain considerable amounts of this vitamin; approximately one third of the normal level ^{186, 186}. In man during times of clinical B₂ avitaminosis

¹⁸⁰ J. R. Klein and H. I. Kohn *J. Biol. Chem.* 136: 177 (1940).

¹⁸¹ A. Emmert and M. van Eckelen *Acta Berol. Neerland. Physiol. Pharmacol. Microbiol.* 7: 129 (1937).

¹⁸² A. Emmert *Ibid.* 8: 116 (1938).

¹⁸³ F. M. Strong, R. E. Feen, Y. B. Moore and H. T. Parsons *J. Biol. Chem.* 137: 363 (1941).

¹⁸⁴ W. Feschara *Z. physiol. Chem.* 229: 103 (1934); 232: 101 (1935).

¹⁸⁵ R. Kuhn, H. Kaltschmitt and T. Wagner-Jauregg *Ibid.* 232: 36 (1935).

¹⁸⁶ F. V. Anco *Arkiv. Arm. Mineral. Geol.* A12: No. 3 (1935).

no substantial decrease of the riboflavin content in blood and in muscles could be observed.¹⁸⁷ In rats¹⁸⁸ and in dogs,¹⁸⁹ riboflavin deficiency causes a decrease of the tissue level of this vitamin. The amount of the riboflavin adenine dinucleotide also decreases during times of low riboflavin intake and increases again upon administration of the vitamin (experiments with rats¹⁹⁰). Thus, the xanthine oxidase activity in the livers of riboflavin depleted rats is only a small percentage of the activity in rats on an adequate riboflavin intake, as measured by the rate of oxygen consumption.¹⁹¹

Riboflavin is like all the other vitamins secreted in the milk, where it is found predominantly in the free form. In human milk, riboflavin is said to occur also to a small extent in combination with a protein.¹⁹²

The fundamental action of riboflavin in living tissue is to take part in enzyme systems which regulate cellular oxidations. These enzyme systems have already been described. They take part in the general carbohydrate metabolism (fermentation and glycolysis). Riboflavin is also involved in the absorption of carbohydrates from the intestines by phosphorylation. Thus glucose and galactose are rapidly absorbed only in the presence of riboflavin.¹⁹³ Riboflavin is connected to a certain extent with the fat metabolism.¹⁹⁴ Increased fat content of the livers has been observed in hens and dogs which died of riboflavin insufficiency.¹⁹⁵ Furthermore, it has been demonstrated¹⁹⁷ that the symptoms of a vitamin B₂ deficiency in rats increase in severity upon administration of increased amounts of fats. On the other hand, when increased doses of riboflavin are given together with increased amounts of fat no deleterious effect is observed. Riboflavin bears an important relation to the amino acid metabolism since *d* amino-acids are deaminated by an enzyme system, which contains the flavin adenine dinucleotide.

¹⁸⁷ A. F. Axelrod, T. D. Spies and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* **46**, 146 (1941).

¹⁸⁸ I. Vivanco, *Naturwissenschaften* **23**, 306 (1935). R. Kuhn, H. Kaltschmitt and T. Wagner Jauregg, *Z. physiol. Chem.* **232**, 36 (1935). J. Croen and J. W. Schuyt, *Arch. néerland. physiol.* **23**, 271 (1938).

¹⁸⁹ H. F. Frazer, N. H. Topping and H. Isbell, *U. S. Pub. Health Service Pub. Health Repts.* **55**, 80 (1940).

¹⁹⁰ S. Ochoa and R. J. Rossiter, *Biochem. J.* **33**, 2008 (1939).

¹⁹¹ A. F. Axelrod and C. A. Elvehjem, *Proc. Am. Soc. Biol. Chem.* **1941**, VI.

¹⁹² P. Ellinger and W. Koschka, *Nature* **133**, 553 (1934).

¹⁹³ L. Laszt and F. Verzár, *Biochem. Z.* **292**, 159 (1935). M. J. Lowitz and I. Verzer, *Biochem. J.* **292**, 189 (1937).

¹⁹⁴ E. W. McHenry and G. Gavin, *J. Biol. Chem.* **125**, 63 (1938).

¹⁹⁵ S. Lepkovsky, L. W. Taylor, T. H. Jukes and H. J. Almquist, *Histochem.* **11**, 559 (1938).

¹⁹⁶ W. H. Sebrell and R. H. Onstott, *U. S. Pub. Health Service Pub. Health Repts.* **53**, 83 (1938).

H. R. Street and G. R. Cowgill, *Am. J. Physiol.* **125**, 323 (1939).

¹⁹⁷ G. J. Manneflug, M. A. Lipton and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* **46**, 100 (1941).

Riboflavin in the free form plays an important role in the vision mechanism in the retina¹⁹⁸ Light converts riboflavin into a 'photo compound' of unknown structure, which process seems to have some bearing on the stimulation mechanism of the optical nerve The primary 'photo compound' is extremely sensitive In the absence of oxygen it is destroyed in the presence of oxygen it is reconverted into riboflavin (Theorell) The mechanism of the riboflavin action in the retina is especially well understood for dim light since light of short wave length is converted into light of longer (yellow green) waves by the fluorescent activity of riboflavin¹⁹⁹ The human eye has a maximum sensitivity for greenish light

The theory has also been advanced that riboflavin takes part in an oxidation system in the cornea²⁰⁰ Since the cornea is avascular, the corneal cells are nourished according to this hypothesis by a specific riboflavin containing enzyme system It is thought that during riboflavin deficiency the body attempts to counteract the missing oxygenation by vascularization

An interesting fact about riboflavin is that the phosphorescence of the glow worm (*Lampyris*) is caused by riboflavin in combination with a special protein²⁰¹ The phosphorescence consists of light of the wave lengths 562-570 m μ It must be assumed that riboflavin plays an important role in some unknown biochemical process in the luminous organs

It is also interesting that the mold *Aspergillus niger* which is free of riboflavin when cultivated under optimum conditions becomes tinted with riboflavin on a medium deficient in magnesium The formation of the pigment is not obtained by restricting the other elements but is increased if a deficiency in magnesium and iron exists simultaneously²⁰²

(b) Relation of Vitamin B to Other Vitamins Hormones and Minerals

Riboflavin bears an obvious relation to other members of the vitamin B group It seems that most of these vitamins act as part of enzyme systems which regulate the carbohydrate fat and amino acid metabolism For example the close relation of riboflavin to the enzyme systems containing nicotinic amide has already been discussed The relation of vita-

¹⁹⁸ A. V. Chase *Science* 83: 484 (1937)

¹⁹⁹ H. Fuler and F. Adler *Arch. v. Chem. Mineral. Geol. Biol.* No. 78 (1934) R. Kuhn and H. K. Ischmitt *Ber.* 68: 386 (1935) F. Adler and H. v. Fuler *Nature* 141: 790 (1938) H. v. Fuler and F. Adler *Z. physiol. Chem.* 223: 10 (1934)

²⁰⁰ O. A. Bensy and S. B. Wolbach *J. Nat. Med.* 69: 1 (1933) R. F. F. Khardt and I. V. Johnson *J. Ophthalmol.* 21: 313 (1939)

²⁰¹ C. Brook *Compt. rend.* 210: 118 (1940)

²⁰² J. Lavollay and F. Laborcy *Ibid.* 208: 1036 (1937)

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¹⁸⁸ F. Vivanco, *Naturwissenschaften* **23**, 306 (1935). R. Kuhn, H. Kaltschmitt and T. Wagner, *Jauregg Z. physiol. Chem.* **232**, 36 (1935). J. Croen and J. W. Schuyt, *Arch. Néerland. physiol.* **23**, 271 (1938).

¹⁸⁹ H. F. Frazer, N. H. Topping and H. Isbell, *U. S. Pub. Health Service Pub. Health Repts.* **55**, 80 (1940).

¹⁹⁰ S. Ochoa and R. J. Rossiter, *Biochem. J.* **33**, 2008 (1939).

¹⁹¹ A. E. Axelrod and C. A. Elvehjem, *Proc. Am. Soc. Biol. Chem.* **1941**, VI.

¹⁹² P. Ellinger and W. Koschura, *Nature* **133**, 553 (1934).

¹⁹³ L. Laszt and F. Verzar, *Biochem. Z.* **292**, 159 (1937). M. Julowicz and L. Verzar, *Biochem. J.* **292**, 187 (1937).

¹⁹⁴ F. W. McHenry and G. Ga. in *J. Biol. Chem.* **125**, 63 (1938).

¹⁹⁵ S. Lepkovsky, L. W. Taylor, T. H. Jukes and H. J. Almquist, *Histog. dis.* **11**, 9 (1938).

¹⁹⁶ W. H. Sebrell and R. H. Oastott, *U. S. Pub. Health Service Pub. Health Repts.* **53**, 83 (1938).

¹⁹⁷ H. R. Street and G. R. Cowgill, *Am. J. Physiol.* **125**, 323 (1939).

¹⁹⁸ G. J. Manneberg, M. A. Lipton and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* **46**, 100 (1941).

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¹⁹⁸ A M Chase *Science* 85 484 (1937)

¹⁹⁹ H v Fuler and E Adler *Arch v Kems Mteral Cel* B11 No 28 (1934) R Kuhn and H Kaltchmitt *Ber* 68 386 (1935) E Adler and H v Fuler *Nature* 141 790 (1938) H v Fuler and E Adler *J phy of Chem* 223 105 (1934)

²⁰⁰ O A Bessey and S B Wolbach *J Exptl Med* 69 1 (1931) R F Eckhardt and L V Johnson *J Ophthalmol* 21 315 (1939)

²⁰¹ G Brooks *Compt rend* 210 2 8 (1940)

²⁰² J Lavollay and F Labory *Ibid* 208 10 6 (1937)

min B₂ to vitamin B₁ may be demonstrated by the fact that riboflavin has an apparent sparing action on thiamin²⁰³

An important relation exists between riboflavin and the adrenal cortex hormone the phosphorylation of riboflavin is carried out with the aid of the adrenal cortex hormone Adrenalectomized animals lose their power of phosphorylating riboflavin Thus, riboflavin phosphoric acid, but not riboflavin, maintains life and growth of adrenalectomized rats²⁰⁴

A number of observations have been made which demonstrate the close relationship of riboflavin to other hormones concerned in the carbohydrate metabolism Rats lose their liver glycogen after injections of thyroxine unless increased amounts of thiamine and riboflavin are given simultaneously²⁰ Depancreatized dogs respond to insulin only in the presence of thiamin and riboflavin, but not in the presence of one of these vitamins alone²⁰⁶

A certain relation of riboflavin to the minimum amount of indispensable magnesium is noted A minimum riboflavin intake increases the minimum magnesium requirements²⁰⁷

15 Avitaminosis and Hypovitaminosis

The basic but unspecific symptoms of a riboflavin deficiency are the same throughout the entire animal kingdom—cessation in growth of young organisms and sudden death of adult organisms

In rats, riboflavin deficiency, even in a relatively early stage, considerably decreases the resistance forces of the organism against infectious diseases for example endemic typhus¹⁰⁸ Growth of young rats is impaired when fed a vitamin B₂ deficient diet and when no riboflavin is administered the animals die Riboflavin deficient animals sometimes show an abnormal intracellular metabolism²⁰⁸ In riboflavin deficient rats an early atrophy of the testis and an involution of the thymus have been observed²⁰⁹ In crease of the diaphragm metabolism in rats is also reported²¹⁰ The development of alopecia (baldness) and cataract (loss of transparency of the

²⁰³ L. N. Ellis and A. Zmachinsky *Science* 86 245 (1937)

²⁰⁴ F. Verzar and L. Laszt *Arch. ges. Physiol. (Pflügers)* 236 693 (1935) 237 476 483 (1936) *Verhandl. Schweiz. Physiol.* 1 VI (1936) *Enzymologia* 3 16 (1937)

²⁰⁵ V. A. Drill *J. Nutrition* 14 355 (1937)

²⁰⁶ R. W. Martin *Z. physiol. Chem.* 248 242 (1937) *Verhandl. deut. Ges. inn. Med.* 50 470 (1938)

²⁰⁷ E. V. Tufts and D. M. Greenberg *J. Biol. Chem.* 122 715 (1938) *Am. J. Physiol.* 121 416 (1938)

²⁰⁸ H. Pinkerton and O. A. Bessey *Science* 89 369 (1939)

²⁰⁹ J. W. Schuyt and J. Croen *Acta Brevia Neerland. Physiol. Pharmacol. Microbiol.* 8 195 (1938)

J. H. Shaw and P. H. Phillips *J. Nutrition* 22 345 (1941)

²¹⁰ A. B. Hastings, J. Muus and O. A. Bessey *J. Biol. Chem.* 120 295 (1935)

lens of eyes) in rats on riboflavin deficient diets is of special interest^{211 212} Certain eye lesions namely, diffuse corneal opacity and vascularization of the cornea, are specific for riboflavin deficiency in rats^{13 214}

Pigs also need riboflavin for normal growth and physical well being²¹⁵ Dogs usually collapse after about 100 days on a riboflavin deficient diet²¹⁶ For chicks and fowls riboflavin is of importance in the production of eggs and for normal hatchability of eggs^{217 218 219 20} Chicks hatched from eggs on a partially riboflavin deficient ration exhibit a characteristic curled toe (nutritional leg paralysis)²²⁰ Besides this slowly developing paralysis an acute paralysis characterized as neuromalacia was observed²²¹ The main peripheral nerve trunks are especially involved and characteristic changes in the myelin have been noticed These severe nerve degenerations occur also in rats²²² Turkey poult develop a typical dermatitis as the result of a riboflavin deficiency²³ In addition to the specific symptoms which are caused by a riboflavin deficiency a premature aging has been observed in all animals studied²²⁴

Riboflavin deficiency in humans of all ages²²⁵ causes the occurrence of specific symptoms Primarily cheilosis an eversion of the lips and in the corners of the mouth has been observed^{6 227} There is also a seborrheic syndrome, a scaly slightly greasy desquamation on a mildly erythematous base in the nasobial fold on the alae nasi in the vestibule of the nose and on the ears A special type of glossitis may develop which is characterized by a purplish red or magenta colored inflammation of the tongue²²⁸ The entire condition is called ariboflavinosis^{2 6}

Riboflavin deficiency in man also causes characteristic ocular symptoms similar to those already described for rats In mild cases a sensation of

¹ P L Day W C Langston and C S O'Brien *Am J Ophthalmol* 14 100 (1931)

² I I Day W J Doby and W C Langston *J Nutrition* 13 389 (1937)

³ M M Ilbader *J Soc Chem Ind* 58 1070 (1939)

⁴ O A Bailey and S B Wolbach *J Exptl Med*, 69 1 (1933) R E Eckert and L V Johnson *Arch Ophthalmol* 21 315 (1933)

⁵ E H Hugh *J Nutrition* 17 97 (1934)

⁶ H R Street and G R Cowell *Am J Physiol* 125 33 (1931)

⁷ S Lepkovsky L W Taylor J H Jukes and H J Almquist *Histochem* 11 1 (1938)

⁸ H J Davis I C Norn and G F Heuser *Poultry Sci* 17 81 87 (1938)

⁹ G F Heuser H S Wilgus and L C Norris *Id* 17 105 (1938)

¹⁰ A F Schumacher and G F Heuser *Id* 18 369 (1939)

¹¹ P H Phillips and H Engel *J Nutrition* 16 41 (1938) *J Nut Sci* 17 417 (1938) I L R Stokstad and I D V Manung *J Nutrition* 16 79 (1938)

¹² J H Shaw and P H Phillips *J Nutrition* 22 345 (1941)

¹³ S Lepkovsky and J H Jukes *J Nutrition* 12 515 (1938)

¹⁴ P I Day and W J Doby *J Biol Chem* 123 8 (1938)

¹⁵ T D Spies W B An R W Valters and N E Huff *Am J Med Sc* 200 617 (1940)

¹⁶ W H Sebrell and R F Butler *US J Health Dev & Pub Health Repts* 53 2282 (1938)

¹⁷ J W Oden I H Oden and W H Sebrell *Id* 54 790 (1933)

¹⁸ H D Kruse V P Sydstricker W H Sebrell and H M Cleckley *Id* 55 157 (1940)

roughness in the eyes and itching and burning are observed²²⁹ which are accompanied by a mild photophobia. In more severe cases a corneal opacity has been noted.²²⁸ Interstitial keratitis (of the cornea) in cases of syphilis improves markedly upon treatment with riboflavin.²⁸ Persons showing subnormal dark adaptation which does not respond to vitamin A treatment may be relieved by riboflavin administration.²³⁰⁻²³¹

Riboflavin treatment is also of importance in diseases caused by multiple vitamin deficiencies, such as pellagra,²³² black tongue²³³ and beriberi. Beneficial results from riboflavin have also been observed in a case of pemphigus.²³⁴

(a) Clinical Test Methods

Vitamin B₂ can be determined in blood, in muscles and in urine. The value of these tests for a detection of a state of a hypovitaminosis or avitaminosis is, however, very limited. While the riboflavin content of tissues from experimental animals, especially from rats, is lowered during times of inadequate vitamin B₂ intake²³⁵⁻²³⁹ this is not the case in human beings as is evident from blood and muscle analysis.²⁴¹ The most promising test is the tissue saturation test but not enough experimental data have accumulated to determine the actual value of this procedure. Blood determinations for riboflavin are best carried out by the *Lactobacillus* test,²⁴² the reliability of which has been established.²⁴³⁻⁴⁴ The riboflavin adenine dinucleotide content of blood can be determined by the *d* amino acid oxidase test.²⁴⁵ In muscle studies the quadriceps femoris muscle²⁴¹ and the *Lactobacillus* test²⁴² are used for the determination of the riboflavin

²²⁹ T. D. Spies, R. W. Vilter and W. F. Ashe, *J. Am. Med. Assoc.* **113**, 931 (1939).

²³⁰ M. S. Kimble and E. S. Gordon, *J. Biol. Chem.* **128**, 141 (1939).

²³¹ P. H. Pock Steen, *Aknephascopia Geneesk. tijdschr. v. Nederl. Indie* **79**, 1986 (1939).

²³² R. W. Vilter, S. Vilter and T. D. Spies, *J. Am. Med. Assoc.* **112**, 470 (1933).

²³³ I. H. Margolis, G. Margolis and S. G. Smith, *J. Nutrition* **17**, 63 (1934).

²³⁴ M. C. Topping and A. F. Knoefel, *J. Am. Med. Assoc.* **114**, 210 (1940).

²³⁵ S. Ochoa and R. J. Rossiter, *Biochem. J.* **33**, 508 (1939).

²³⁶ A. E. Axelrod, H. A. Sober and C. A. Elvehjem, *J. Biol. Chem.* **134**, 749 (1940).

²³⁷ R. Kuhn, H. Kaltschmitt and T. Wagner Jauregg, *Z. physiol. Chem.* **232**, 36 (1936).

²³⁸ J. Groen and J. W. Schuyt, *Arch. néerland. physiol.* **23**, 271 (1938).

²³⁹ E. V. Carlsson and H. C. Sherman, *J. Nutrition* **15**, 57 (1938).

²⁴⁰ H. F. Frazer, N. H. Topping and H. Isbell, *U. S. Pub. Health Service Pub. Health Repts.* **55**, 280 (1940).

²⁴¹ A. E. Axelrod, T. D. Spies and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* **46**, 146 (1941).

²⁴² E. F. Snell and F. M. Strong, *Ind. Eng. Chem. Anal. Ed.* **11**, 346 (1939); *Enzymologia* **6**, 186 (1939). E. F. Snell, F. M. Strong and W. H. Peterson, *Biochem. J.* **31**, 1789 (1937); *J. Am. Chem. Soc.* **60**, 2825 (1938).

²⁴³ R. F. Feeney and F. M. Strong, *Proc. Am. Soc. Biol. Chem.* **1940**, XXXI.

²⁴⁴ F. M. Strong, R. F. Feeney, B. Moore and H. I. Parson, *J. Biol. Chem.* **137**, 363 (1941).

²⁴⁵ J. R. Kretz and H. J. Kohn, *Ibid.* **136**, 177 (1940).

present The amount of riboflavin present in urine can be assayed by the Lactobacillus test²⁴⁴ or by the fluorescence method Urine containing 0.0001 mg or less of riboflavin per cc cannot be used in the latter procedure²⁴⁵ In the *tissue saturation test* the amount of riboflavin excreted through the urine is measured after oral administration of excess doses of the vitamin²⁴⁵

16 Hypervitaminosis

Riboflavin administered in excessive amounts by mouth was found to be non toxic in dogs and rats Following intraperitoneal injection however riboflavin produced death due to kidney concretions^{246 247}

17 Requirements

A regular dietary intake of riboflavin is necessary for all members of the animal kingdom and for some microorganisms From eleven species of lactic acid bacteria investigated, four were found to require riboflavin for growth and acid production The remaining seven did not require an external supply of this vitamin and it has been established that four of these seven species synthesize vitamin B when cultured on a riboflavin free medium^{248 49} Riboflavin also stimulates the growth of propionic acid²⁵⁰ and of luminous²⁵¹ bacteria

The only organism so far reported as actually needing the riboflavin adenine dinucleotide is the larva of mosquitoes Riboflavin itself is not effective²⁵²

In general, the vitamin B₂ requirements of animals are related to body size and weight, to the amount of food ingested to the ambient temperature etc Man needs about 2-3 mg of riboflavin daily^{33 254} The riboflavin allowances recommended by the Food and Nutrition Board National Research Council, will be found on page 613

¹ R Kuhn and P Boulenger *Z physiol Chem* 241 933 (1936) R Kuhn *Altn Hochsch* 17 22 (1938) V Demole *Z Vitaminsforsch* 7 138 (1934)

² K Unna and J G Creslin *J Pharmacol* 76 75 (1942)

³ E C Snell and F M Strong *Enzymologia* 6 186 (1933)

⁴ E B Sill and M Strong and W H Peterson *Biochem J* 31 1793 (1937) ⁵ Orla Jen and N C Otte and A Sogkjer *Zentr Bakt Parasitenk* 11 94 434 447 (1936) H G Wood A A Anderson and C H Werkman *J Bact* 34 137 (1937) *Proc Soc Exptl Biol Med* 36 217 (1937)

⁶ H G Wood A A Anderson and C H Werkman *J Bact* 36 36 36 (1938)

⁷ M Doedoff *Enzymologia* 5 239 (1938)

⁸ W Trugan and V Subbier *Biol Bull* 75 7 (1938)

⁹ W Stapp J Kühn and A H Schöeder *Die Vitamine* Stuttgart 1937 p 7

¹⁰ W H Sebrell R B Butler J C Woolly and H Ishii *L S Pub Health Service Pub Health Reps* 36 510 (1941)

Chickens need 230-245 γ per 100 g. of diet to maintain normal hatch ability^{55 256} Baby chicks need increased amounts²⁵⁶ Turkeys require about 25% more riboflavin than chickens

Some animals apparently need no riboflavin or only a very small amount in their food Microorganisms which synthesize this vitamin live in the rumen of sheep²⁵⁷ and of cattle^{258 259} and make the vitamin available to the animal

⁵⁵ H. J. Davis, I. C. Norris and C. I. Heuser *Poultry Sci.* **17**, 81-87 (1938)

⁵⁶ C. I. Heuser, H. S. Walpus and I. C. Norris *Ibid.* **17**, 103 (1938)

⁵⁷ I. W. McElroy and H. Goss *J. Biol. Chem.* **130**, 437 (1939)

⁵⁸ I. W. McElroy and H. Goss *Ibid.* **133**, 1 XV (1940)

⁵⁹ M. I. Wegner, A. N. Booth, C. A. Flvehjem and K. B. Hart *Proc. Soc. Exptl. Biol. Med.* **45**, 769 (1940)

VITAMIN B₆—
PYRIDOXIN

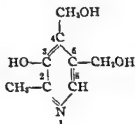
VITAMIN B₆—PYRIDOXIN

1 Nomenclature and Survey

Names

- Pyridoxin Term suggested by György¹ and generally adopted in the United States
- Adermin European name
- Vitamin B₆ of György
- Anti acrodynia rat factor
- Rat antidermatitis factor
- Yeast eluate factor^{2, 3}
- Factor I of Lepkovsky⁴
- Factor Y of Chick⁵
- Vitamin H of Richardson and Hogan⁶ and of Booher⁷
- Complimentary factor⁸

Chemical formula



Chemical name

3-Hydroxy-4,5-dihydro-2-methylpyridine

Empirical formula

C₈H₁₁O₃N

¹ P. György and R. L. Eckhardt *Nature* 144, 512 (1939)

² C. E. Edgar and T. F. Macrae *Biochem. J.* 31, 886 (1937)

M. M. El-Sadr, T. F. Macrae and C. E. Work *Ibid.* 33, 611 (1939)

³ S. Lepkovsky, T. H. Jukes and M. E. Kruse *J. Biol. Chem.* 115, 557 (1936)

⁴ H. Chick, A. M. Copping and M. H. Roscoe *Biochem. J.* 24, 1748 (1930)

⁵ L. R. Richardson and A. C. Hogan *Missouri Agr. Exptl. Station Research Bull. No. 241* (1936) *Science* 83, 17 (1936)

⁶ L. I. Booher *J. Biol. Chem.* 119, 273 (1937)

The term vitamin H was used in early days of vitamin research to designate vitamin B₆. The letter H has also been used for a trout growth factor¹⁰. In present day literature the term vitamin H is reserved for the curative factor for egg white injury (see page 469).

⁷ P. György, R. Kuhn and T. Wagner-Jauregg *Naturwissenschaften* 21, 561 (1933) *Klin. Wochenschr.* 12, 1241 (1933)

⁸ A. C. Hogan *The Vitamin*, Am. Med. Assoc., Chicago, 1939, p. 273

⁹ C. M. McCay, F. C. Bag and W. E. Dill *Science* 67, 249 (1928)

2 Chronology

- 1926 GOLDBERGER and LILLIE¹¹ reported the occurrence of a characteristic dermatitis, called acrodynia, on rats fed a diet deficient in vitamin B₂.
- 1932 OHDAKE¹² in Japan isolated a compound of the formula C₈H₁₁O₂N HCl from rice polishings but failed to recognize its vitamin character.
- 1934 GYÖRGY¹³ established the difference of the rat pellagra preventive factor from vitamin B₂ (and vitamin B₄) and called the new vitamin B₆.
- 1938 The isolation of the pure crystalline vitamin B₆ was announced independently by five different groups, namely by LEPKOVSKY,¹⁴ KERESZTESY and STEVENS,¹⁵ GYÖRGY,¹⁶ KUHN and WENDT¹⁷ and ITIBA and MITI.¹⁸
- 1939 The chemical structure was elucidated and vitamin B₆ was synthesized independently by two groups of workers—by KUHN, WESTPHAL, WENDT and WESTPHAL in Germany and by KERESZTESY, STEVENS, HARRIS, STILLER and FOLKERS in the U. S. A.

3 Occurrence

Vitamin B₆ appears to be very widely distributed over the entire animal and plant kingdom. Reliable systematic studies as to the relative quantities in various foodstuffs are scarce. Yeast and rice polishings are especially rich in vitamin B₆. Seeds and cereals, for example, wheat and maize, are good sources,¹⁹ especially the germs and the integuments.²⁰ Molasses,²¹ fish and fish livers²² and mammalian livers contain moderate amounts,^{21, 23} and milk, egg yolks, lettuce, spinach, etc., contain small amounts of vitamin B₆.

Vitamin B₆ occurs in animal and in plant tissues, for example, in yeast²⁴ and in fish muscle,²⁵ only to a small extent in the free form. The majority (60–80%) is chemically bound to protein⁴ and to starch.²⁶

¹¹ J. Goldberger and R. D. Lillie, *U. S. Pub. Health Service Lab. Health Rept.* 41, 1075 (1926).

¹² S. Ohdake, *Bull. Agr. Chem. Soc. Japan* 8, 111 (1932); P. W. Wiard, *Nature* 142, 1158 (1938).

¹³ P. György, *Nature* 133, 498 (1934); *Biochem. J.* 29, 741, 760, 767 (1935).

¹⁴ S. Lepkovsky, *Science* 87, 169 (1938).

¹⁵ J. C. Keresztesy and J. R. Stevens, *Proc. Soc. Exptl. Biol. Med.* 38, 64 (1938).

¹⁶ P. György, *J. Am. Chem. Soc.* 60, 983 (1938).

¹⁷ R. Kuhn and G. Wendt, *Ber.* 71, 780, 1118 (1938).

¹⁸ A. Itiba and K. Miti, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)* 34, 623 (1938).

¹⁹ H. A. Schneider, J. K. Ascham, B. R. Platz and H. Steenbock, *J. Nutrition* 18, 99 (1939).

²⁰ A. M. Copping, *Biochem. J.* 30, 849 (1936).

²¹ A. van Schoor, *Merck's Jahresberichte* 52, 7 (1938).

²² G. I unde and H. Kringstad, *Biochem. J.* 32, 708 (1938).

²³ C. I. Edgar, M. M. El Sadr and T. F. Macrae, *Ibid.* 32, 2725 (1938).

²⁴ R. Kuhn and G. Wendt, *Be.* 71, 780 (1938).

²⁵ T. W. Birch and P. György, *Biochem. J.* 30, 304 (1936).

²⁶ H. Chick, M. M. El Sadr and A. N. Worden, *Biochem. J.* 34, 595 (1940).

4 Isolation

As has been pointed out in the previous section vitamin B₆ occurs to a great extent bound to a protein. From this symplex, vitamin B₆ cannot be separated by dialysis. The symplex can however be split by heating^{27 28 29} or by enzymatic hydrolysis.³⁰

The free vitamin B₆ is extracted with water or with organic solvents such as ether, propanol or butanol.^{31 32} The latter solvents extract less by products than does water, but continuous extraction is necessary due to the water solubility of the vitamin.

From neutral or acidified water solutions, vitamin B₆ can be adsorbed on charcoal and fuller's earth.³³ The adsorption on fuller's earth is greatly influenced by the pH of the solution. At pH 5-6 vitamin B₆ is not quantitatively adsorbed even after three consecutive adsorptions. At pH 1 factors other than B₆ are also adsorbed.³⁴ The elution is carried out with barium hydroxide³⁵ or with butyl alcohol.³⁶ Vitamin B₆ is quantitatively adsorbed on zeolite and can be subsequently eluted with 10% potassium chloride.³⁷ Inert material can be removed with acetone ethyl alcohol ethyl acetate platinum chloride etc. The vitamin is precipitated by a number of acids, such as by sulfuric acid, phosphotungstic acid, silicotungstic acid, Reinecke's acid etc. By repeated precipitations the pure vitamin B₆ can be obtained in the form of various salts, such as the hydrochloride. The free base is prepared therefrom by treatment with silver salts.³⁸

5 Properties

Vitamin B₆, as a free base, is a colorless crystalline powder, has a slightly bitter taste and melts at 160° C.^{38 39} It is readily soluble in water, in acetone and in alcohol, and slightly soluble in ether and chloroform. Vitamin B₆ dialyzes easily. It crystallizes in the form of various salts.

²⁷ R. Kuhn and G. Wendt *Ber* 71 780 (1938)

²⁸ E. M. Lantz *New Mexico Ag. Exptl. Station Bull.* No. 268

²⁹ H. Chalk, M. M. El-Sadr and A. N. Worden *Biochem. J.* 34 512 (1940)

³⁰ T. W. Birch and P. György *Ibid.* 30 304 (1936)

³¹ J. V. Scudl, H. F. Koonce and J. C. Keresztessy *Proc. Am. Physiol. Soc.* 1940 163 *Proc. Soc. Exptl. Biol. Med.* 43 118 (1940)

³² R. D. Greene *J. Biol. Chem.* 130 513 (1939)

³³ T. W. Birch and P. György *Biochem. J.* 30 304 (1936)

³⁴ G. Lunde and H. Kringstad *J. Nutrition* 19 321 (1940)

³⁵ N. Halliday and H. M. Evans *J. Biol. Chem.* 118 205 (1937)

³⁶ O. A. Emerson, A. Mohammad, O. H. Emerson and H. M. Evans *Ibid.* 124 377 (1938)

³⁷ J. V. Scudl, H. F. Koonce and J. C. Keresztessy *Proc. Am. Physiol. Soc.* 1940 163 *Proc. Soc. Exptl. Biol. Med.* 43 118 (1940)

³⁸ A. Iitaba and K. Mitu *Sci. Papers Inst. Phys. Chem. Research (Tokyo)* 34 1014 (1938)

³⁹ J. C. Keresztessy and J. P. Stevens *J. Am. Chem. Soc.* 60 1267 (1938)

for example as hydrochloride, m p 204–206° C³⁹ (with decomposition), and as picrate. The hydrochloride is soluble in water (1 g in 4 cc water) and in alcohol (1 g in 90 cc alcohol) and somewhat soluble in acetone. The aqueous solution has a pH of about 3.2. Both the free vitamin B₆ and its hydrochloride sublime readily³⁹. The hydrochloride is the form in which this vitamin is marketed. It is a white odorless powder with salty taste. It is stable to heat, concentrated hydrochloric acid and alkali, but is destroyed by light.

Vitamin B₆ is optically inactive. It exhibits a typical ultraviolet absorption spectrum which changes markedly with a change of hydrogen ion concentration. The spectra of the vitamin in aqueous solution between pH 4 and 6.75 are shown in Fig. 11.

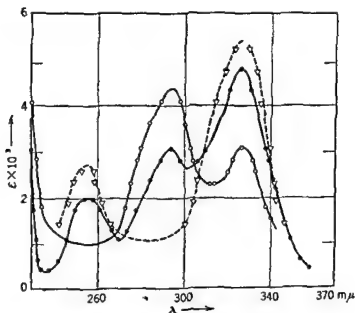


Fig. 11—Absorption spectra of vitamin B₆ at ○ pH 4
● pH 5.1 ▽ pH 6.75 (E. T. Stiller, J. C. Keresztesy, and
J. R. Stevens)

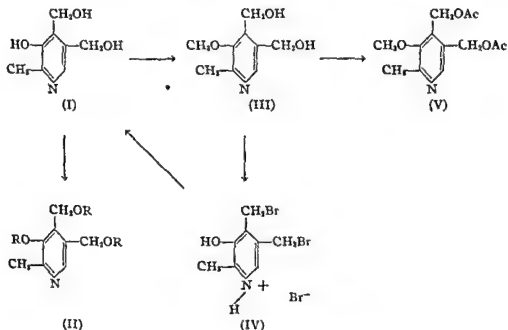
6 Constitution

Vitamin B₆ (I) has the empirical formula C₈H₁₁O₃N and forms salts easily with acids, such as hydrochloric acid, picric acid, etc. The hydrochloride yields an orange-red color with ferric chloride and couples with diazonium salts, for example with diazotized sulfanilic acid, which properties suggest the presence of a phenolic or enolic hydroxyl group.⁴⁰

⁴⁰ R. Kuhn and G. Wendt, *Ber.* 71, 1118 (1938).

All three oxygens of the molecule are present in the form of hydroxyl groups. A triacetate which can be distilled^{41, 42} and a tribenzoate⁴³ (II) can be prepared. An estimation of active hydrogen atoms showed the presence of three in the molecule⁴³.

Upon reaction with diazomethane a compound, $C_9H_{13}O_3N$, is obtained which does not give a color reaction with ferric chloride and which does not couple with diazotized sulfanilic acid. Treatment of this compound with hydriodic acid yields methyl iodide. Quantitative estimation of these properties led to the conclusion that a mono methyl ether of vitamin B_6 (III) is produced by diazomethane⁴⁴. Vitamin B_6 methyl ether is split upon reaction with hydrobromic acid to a hydrobromide of a dibromide, $C_9H_{10}ONBr_3$ (IV), which yields vitamin B_6 by treatment with silver acetate⁴⁵.



Besides the methyl ether, diazomethane produces by reaction with vitamin B_6 the N methyl vitamin B_6 ⁴⁶ which yields a color reaction with ferric chloride.

⁴¹ R. Kuhn and G. Wendt *Ber.* 71 780 (1938)

⁴² A. Itaba and K. Mitani *Sci. Papers Inst. Phys. Chem. Research (Tokyo)* 35 73 (1938) 36 1 (1939)

⁴³ E. T. Still and J. C. Kereziya and J. R. Steven *J. Am. Chem. Soc.* 61 1237 (1939)

⁴⁴ R. Kuhn and G. Wendt *Ber.* 71 1574 (1938)

⁴⁵ R. Kuhn and G. Wendt *Ibid.* 72 311 (1939)

⁴⁶ A. Itaba and K. Mitani *Sci. Papers Inst. Phys. Chem. Research (Tokyo)* 35 73 (1938) 36 1 (1939)

The methyl ether of vitamin B₆ is converted by the action of acetic anhydride in pyridine into a diacetyl methyl ether (V). Thus it is concluded that of the three hydroxyl groups one is phenolic (or enolic) and the other two are aliphatic hydroxyl groups. All active hydrogen atoms are accounted for and no farther active hydrogen atoms could be detected in the diacetyl methyl ether. Therefore, it must be concluded that the nitrogen is present in a ring.⁴⁷

The position of the phenolic hydroxyl group was determined, first, by application of the Folin Denis phenol reagent⁴⁸ which produced a color with vitamin B₆ and with β hydroxy pyridine, but not with α and γ hydroxy pyridine,⁴⁹ and second, by investigation of the ultraviolet absorption spectrum which proved to be similar to that of β hydroxy pyridine.^{49, 50} Upon application of the color test with 2,6 dichloro quinone chlorimide vitamin B₆ gives a blue color,⁵⁰ which effect proves, according to Gibbs,⁵¹ that the *p* position to the hydroxyl group is not substituted.

Vitamin B₆ methyl ether does not react with lead tetra acetate, which indicates that the two aliphatic hydroxyl groups are not in α β position.⁴⁹ Oxidation in neutral aqueous solution with potassium permanganate in an amount corresponding to two atoms of oxygen converts vitamin B₆ methyl ether (III) into a lactone (VI), which indicates that the two aliphatic hydroxyl groups are either in 1,4- or in 1,5 position to each other.^{49, 52} Upon further oxidation of vitamin B₆ methyl ether with barium permanganate, four atoms of oxygen are taken up. The reaction product is a dicarboxylic acid (VII)^{52, 53, 54, 55} which contains all the carbon atoms of the vitamin methyl ether. By treatment of this dicarboxylic acid with acetic anhydride the dicarboxylic acid is dehydrated yielding the anhydride (VIII),⁵⁴ indicating that the two carboxyl groups are vicinal. Indication of the same fact is given by the fusion of the dicarboxylic acid with resorcinol⁵⁶ which yielded a phthalein having a greenish yellow fluorescence. Decarboxylation of the dibasic acid, by heating the disodium salt with calcium hydroxide, yielded a hydroxy picoline (XI).⁵⁵

Vitamin B₆ methyl ether on oxidation with potassium permanganate in alkaline solution takes up seven atoms of oxygen and forms a tricarboxylic

⁴⁷ R. Kuhn and G. Wendt *Ber* 71 1534 (1938)

⁴⁸ O. Folin and W. Denis *J. Biol. Chem.* 12 239 (1912) 22 305 (1915)

⁴⁹ R. Kuhn and G. Wendt *Ber* 72 305 (1939)

⁵⁰ E. T. Stiller, J. C. Keresztesy and J. R. Stevens *J. Am. Chem. Soc.* 61 1237 (1939)

⁵¹ H. D. Gibbs *J. Biol. Chem.* 72 649 (1927) E. J. Theriault *Ind. Eng. Chem.* 21 343 (1929)

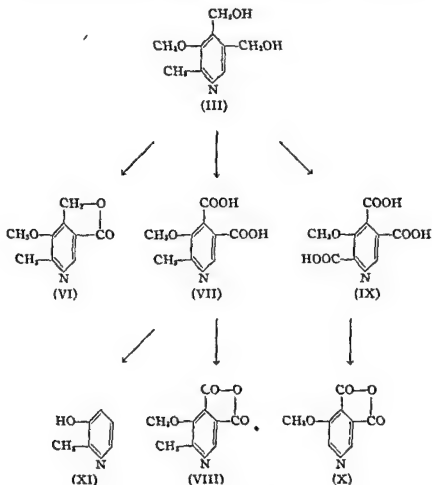
⁵² A. Itaba and K. Miti *Sci. Papers Inst. Phys. Chem. Research (Tokyo)* 35 73 (1938) 36 1 (1939)

⁵³ R. Kuhn, H. Andersag, K. Westphal and G. Wendt *Ber* 72 309 (1939)

⁵⁴ R. Kuhn, G. Wendt and K. Westphal *Ibid.* 72 310 (1939)

⁵⁵ E. T. Stiller, J. C. Keresztesy and J. R. Stevens *J. Am. Chem. Soc.* 61 1237 (1939)

acid (IX) without the loss of carbon atoms⁵⁶ This tricarboxylic acid yields an anhydride of a dicarboxylic acid (X) while simultaneously losing one mol of carbon dioxide⁵⁶ The tricarboxylic acid yields with ferrous sulfate a reddish color that is characteristic for pyridine α -carboxylic acids



This color reaction is not given by the dicarboxylic acids (VII) and (X). This proves that the carboxyl group lost by anhydration of the tricarboxylic acid was in α position to the ring nitrogen⁵⁶ and that this carboxyl group originated from a methyl group⁵⁷. The existence of the methyl group has also been shown by oxidation of the vitamin chlorohydrate with chromic acid in sulfuric acid whereby acetic acid is obtained

⁵⁶ R. Kuhn and C. Wendt *Ber.* 72 300 (1939)

⁵⁷ R. Kuhn, H. Andersag, K. Westphal and C. Wendt *ibid.* 72 309 (1939)

7 Synthesis

Two different methods have been described for the synthesis of vitamin B₆, one of which is a complete synthesis building up the pyridine nucleus from small aliphatic molecules. The other synthesis is a partial degradation of a higher molecular compound to the pyridine derivative of the constitution of vitamin B₆.

(a) *The Complete Synthesis of Harris and Folkers^{55 59} and of Mori and Makino⁶⁰*

By a series of seven reactions vitamin B₆ has been synthesized as follows

Step 1 Cyano acetamide (I) is condensed with ethoxy acetyl acetone (II) in the presence of piperidine to yield 3 cyano 4 ethoxy methyl 6 methyl 2 pyridone (III)

Step 2 By nitration of the reaction product of step 1, 3 cyano 4 ethoxy methyl 5 nitro 6 methyl 2 pyridone (IV) is obtained

Step 3 Chlorination converts the last mentioned compound (IV) into 2 methyl 3 nitro 4 ethoxy methyl 5 cyano 6 chloro pyridine (V)

Step 4 Partial catalytic hydrogenation of (V) yields 2 methyl 3 amino-4 ethoxy methyl 5 cyano 6 chloro pyridine (VI)

By a less attractive series of reactions the amino chloro compound (VI) can be obtained from the nitro pyridone (IV) by reduction of the nitro group to the amino pyridone (X) followed by chlorination

Step 5 The cyano chloro pyridine (VI) is catalytically hydrogenated to 2 methyl 3 amino 4 ethoxy methyl 5 amino methyl pyridine (VII)

A modification of this step consists in first acetylating the 3 amino group of (VI) to give (XI) followed by hydrogenation to remove the chlorine and to convert the cyano group in the amino methyl group (XII). Finally the acetyl groups of the 3 amino group are split off by hydrolysis

Step 6 The 4 ethoxy group is now saponified with dilute hydrochloric acid to yield the dihydrochloride of 2 methyl 3 amino 4 hydroxy methyl 5 amino methyl pyridine (VIII)

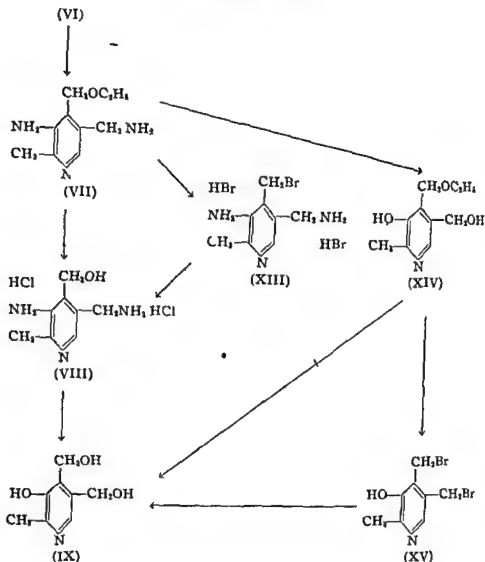
If this reaction is carried out with hydrobromic acid, the dihydrobromide of a 4 bromo methyl compound (XIII) is obtained, which must be saponified to give (VIII)

Step 7 The last step of this synthesis consists in diazotization of the diamine (VIII) to yield vitamin B₆ (IX)

⁵⁵ S. A. Harris and F. Folkers *J. Am. Chem. Soc.* **61** 1245 (1939) ⁵⁹ S. A. Harris, E. T. Stiller and F. Folkers *Ibid.* **61** 1247 (1939)

⁵⁸ S. A. Harris and F. Folkers *Ibid.* **61** 1307 (1939)

⁶⁰ S. Mori and K. Makino *Insulinologia* **7** 383 (1939)



acid, hydrobromic acid is used, the dibromide (XV) is obtained, which requires another step to yield finally vitamin B₆.

(b) Synthesis by Degradation of Isoquinoline

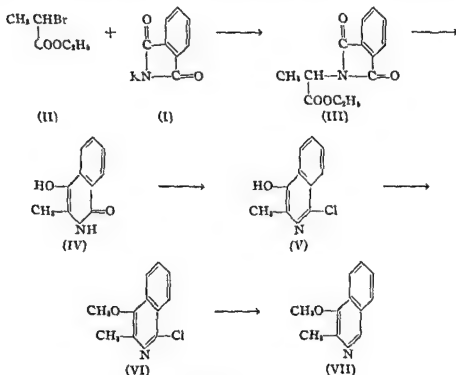
The principle of this synthesis has independently been used by two groups of workers, by Kuhn and co workers⁶¹ in Germany and by Itaba and Mitu⁶² in Japan, and consists of the oxidative degradation of 2 methyl 3 methoxy isoquinoline to 2 methyl 3 methoxy pyridine-4 5 dicarboxylic acid. The isoquinoline derivative is prepared⁶³ by condensation of potas

⁶¹ R. Kuhn, K. Westphal, O. Wendt and O. Westphal, *Naturwissenschaften* 27, 469 (1939).

⁶² A. Itaba and K. Mitu, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)* 36, 173 (1939).

⁶³ S. Gabriel and J. Colman, *Ber.* 33, 988 (1900).

sum phthalimide (I) with α bromo propionic acid ester (II) to yield α phthalimide propionic acid ester (III) which upon saponification gives 2 methyl 3 hydroxy isocarbostyryl (IV). Chlorination yields the chloro compound (V) which is converted into the *O* methyl ether (VI) by methylation with methyl iodide. The latter compound upon treatment with tin and hydrochloric acid yields the 2 methyl 3 methoxy isoquinoline (VII).

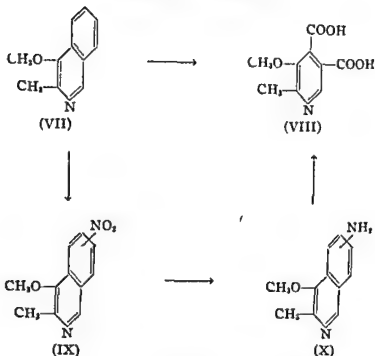


The 2 methyl 3 methoxy isoquinoline (VII) is converted into 2 methyl 3 methoxy pyridine 4 5 dicarboxylic acid (VIII) either by direct oxidation in alkali solution with permanganate⁶⁴ or by nitration to a Bz nitro compound (IX) followed by reduction to a Bz amino compound (X) which is then oxidized with permanganate to yield the dicarboxylic acid (VIII).⁶⁵

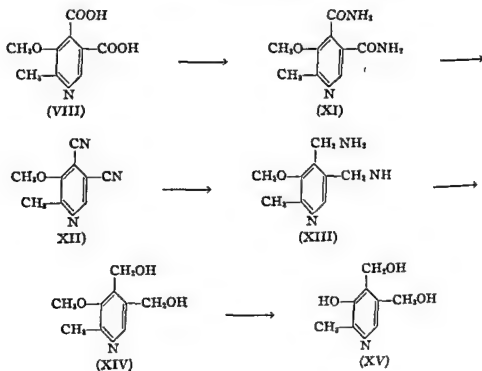
The 2 methyl 3 methoxy pyridine-4 5 dicarboxylic acid (VIII) is converted through its diamide (XI) into 2 methyl 3 methoxy 4 5 dicyano pyridine (XII), which by catalytic hydrogenation yields the 2 methyl 3 methoxy 4 5 diamino methyl pyridine (XIII). The latter upon reaction with nitrite gives the vitamin B₆ *O* methyl ether (XIV). The methyl

⁶⁴ A. Itaba and K. Mitsu, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)* 36, 173 (1939).

⁶⁵ R. Kuhn, K. Westphal, C. Wendt and O. Westphal, *Naturwissenschaften* 27, 469 (1939).



ether can be converted into the vitamin B₆ (XV) according to one of the previously mentioned methods



8 Industrial Methods of Preparation

Pure vitamin B₆ obtained by extraction of animal or plant material has never appeared on the market. It has, however, been commercially available in the form of yeast or liver concentrates in mixture with several other members of the vitamin B complex. In the future, extracts of this type will probably be prepared primarily as a source of the less well known members of the vitamin B group.

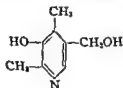
Vitamin B₆ is commercially available in the pure crystalline form, synthesized according to both methods outlined in the previous section dealing with the synthesis of vitamin B₆.

9 Biogenesis¹

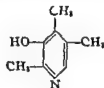
The biogenesis of vitamin B₆ is unknown. Theories pertaining to the biogenesis of this vitamin have not been suggested.

10 Specificity

Vitamin B₆ apparently owes its physiological action to the molecule as an entity. Compounds of very similar structure and simple derivatives of vitamin B₆ are inactive. The di- and triacetate are fully active^{68, 6} probably because the organism is able to hydrolyze these esters. The benzoate, however, is inactive. The same is true for the methyl ether,^{67, 69} which, however, shows some activity in a concentration that corresponds to about 500 times the concentration of the free vitamin.⁶⁹ 4-Desoxy vitamin B₆ (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine (I)) appears to be active in 50 times the concentration of vitamin B₆,^{69, 70} and 4,5-bis-desoxy vitamin B₆ (2,4,5-trimethyl-3-hydroxypyridine (II)) appears to be inactive.⁶⁹ A great number of other pyridine derivatives have been tested⁶⁹ but no active compound has been found.



(I)

4-Desoxy vitamin B₆

(II)

4,5-Bis-desoxy vitamin B₆

¹ R. Kuhn and G. Wendt *Ber.* 71 1118 (1938)

² K. Unna *Proc. Soc. Exptl. Biol. Med.* 43 1 2 (1940)

³ R. Kuhn and G. Wendt *Ber.* 71 1534 (1938)

⁴ L. F. Möller *Z. phys. i. Chem.* 260 246 (1939)

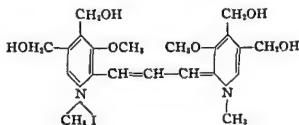
⁵ F. F. Möller, O. Fim, F. Jung and T. Möll *Naturwissenschaften* 27 278 (1939)

11 Determination

(a) Chemical Methods

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Cyanine Dye Test⁷² This test can be applied only to the methyl ether of quaternary vitamin B₆ salt. Therefore, vitamin B₆ must, first, be converted into the methyl ether with diazo methane, and, second, into the iodo methyl pyridinium- or a similar pyridinium compound. These two reactions can be carried out in a yield of 30–40%. The methyl ether pyridinium salt, upon heat treatment with chloroform and potassium hydroxide, yields a violet dye of the structure of a carbo pyridine cyanine dye (absorption maxima at 599 and 555 mμ in alcohol chloroform). This test is believed to be quite specific to the vitamin since no other α picoline derivatives are known to occur in plants or animals. By the use of this method 0.8 mg of pyridoxin can be determined.



Gibb's Phenol Indophenol Test This test has been adapted to the quantitative colorimetric determination of vitamin B₆.^{73 74} A blue color develops when a vitamin B₆ solution is mixed with a veronal buffer (pH 7.6) and a butanol solution of 2,6 dichloroquinone chloroimide. The color has an absorption maximum at 660 mμ and is measured in a colorimeter or spectrophotometer.

⁷¹ O. Folin and W. Denis, *J. Biol. Chem.* 12, 239 (1912); 22, 305 (1915).

⁷² R. Kuhn and I. Löw, *Ber.* 72, 1453 (1939).

⁷³ J. V. Scudé, H. F. Koonen and J. C. Keresztesy, *Proc. Am. Physiol. Soc.* 1940, 163. *Proc. Soc. Exptl. Biol. Med.* 43, 118 (1940).

⁷⁴ J. V. Scudé, K. Unna and W. Antopol, *J. Biol. Chem.* 135, 371 (1940).

Ferric Chloride Method^{7 78} This method has proved to be of value in the estimation of vitamin B₆ in rich sources. The red brown color developed by the unknown material is compared with those of standards.

(b) Biological Methods

Yeast Growth Test Since it has been observed that vitamin B₆ stimulates the growth of yeast, a method of determining this vitamin by the rate of growth of yeast has been proposed.⁷⁷

Bacteria Test *Streptobacterium plantarum* (*Bacterium Acetyl Cholini* Heil 10 S) responds in growth and production of acid to the amount of vitamin B₆ present. The increase in growth is measured with a nephelometer.^{78 79}

Rat Growth Test In this proposed test the growth rate increase of rats, following the administration of vitamin B₆, is determined.^{80 81 82 83} Since the decline of the growth rate is not specific for a vitamin B₆ deficiency, this test often gives unsatisfactory results.⁸³

Acrodynia Rat Test Vitamin B₆ is assayed comparatively on the basis of the percentage incidence of the typical acrodynia type dermatitis in rats.^{84 85 86 87 88 89 90 91 92} in either prophylactic or curative assay procedures.

12 Standard

One rat unit vitamin B₆ = 10 γ , is defined as the amount necessary per day to cure or prevent the typical symptoms of avitaminosis (Gyorgy).⁹³

⁷⁸ J. C. Keresztesy and J. P. Stevens *J. Am. Chem. Soc.* 60 1267 (1938).

⁷⁹ R. D. Greene *J. Biol. Chem.* 130 513 (1939).

⁸⁰ A. S. Schultz, L. Atkin and C. N. Frey *J. Am. Chem. Soc.* 61 1931 (1939).

⁸¹ E. F. Möller *Z. physiol. Chem.* 254 280 (1938).

⁸² E. F. Möller, O. Fima, F. Jung and T. Moll *Naturwissenschaften* 27 278 (1939).

⁸³ M. K. Dmick and C. B. Schreffler *J. Nutrition* 17 23 (1939).

⁸⁴ R. Kuhn and G. Wendt *Z. physiol. Chem.* 256 127 (1938).

⁸⁵ C. E. Edgar, M. M. El Sadr and T. F. Macrae *Biochem. J.* 32 2207 (1938).

⁸⁶ T. W. Conger and C. A. Elvehjem *J. Biol. Chem.* 138 555 (1941).

⁸⁷ I. György and R. F. Eckardt *Biochem. J.* 34 1143 (1940).

⁸⁸ R. C. Bender and G. C. Supplee *J. Am. Chem. Soc.* 59 1178 (1937).

⁸⁹ I. György *Biochem. J.* 29 760 (1935).

⁹⁰ G. Lunde and H. Kringstad *Ibid.* 32 708 (1938).

⁹¹ H. I. C. Wilson and G. K. Roy *Indian J. Med. Research* 25 879 (1938).

⁹² H. A. Schneider, J. K. Ascham, B. R. Platz and H. Steenbock *J. Nutrition* 18 99 (1939).

⁹³ N. H. Lloyd and H. M. Evans *J. Biol. Chem.* 118 250 (1937).

⁹⁴ M. K. Dmick and C. B. Schreffler *J. Nutrition* 17 73 (1937).

⁹⁵ E. J. Reedman, W. L. Simpson and K. Unna *Proc. Soc. Exptl. Biol. Med.* 43 112 (1940).

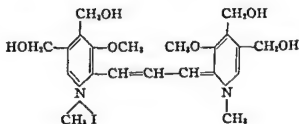
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⁷¹ O. Folin and W. Denis, *J. Biol. Chem.* 12: 239 (1917); 22: 305 (1915).

⁷² R. Kuhn and I. Löw, *Ber.* 72: 1453 (1939).

⁷³ J. V. Scudl, H. F. Koones and J. C. Keresztes, *Proc. Am. Physiol. Soc.* 1940: 163. *Proc. Soc. Exptl. Biol. Med.* 43: 118 (1940).

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⁷⁸ E. F. Möller *Z physiol Chem* 254 280 (1938)

⁷⁹ E. F. Möller, O. Fima, F. Jung and T. Moll *Naturwissenschaften* 27 228 (1939)

⁸⁰ M. K. Dimick and C. B. Schreffler *J Nutrition* 17 3 (1939)

⁸¹ R. Kuhn and G. Wendt *Z physiol Chem* 256 127 (1938)

⁸² C. E. Edgar, M. M. El Sadr and T. F. Macrae *Biochem J* 32 2207 (1938)

⁸³ T. W. Conger and C. A. Elvehjem *J Biol Chem* 138 355 (1941)

⁸⁴ P. György and R. F. Eckardt *Biochem J* 34 1143 (1940)

⁸⁵ R. C. Under and G. C. Supplee *J Am Chem Soc* 59 1178 (1937)

⁸⁶ P. György *Biochem J* 29 700 (1935)

⁸⁷ G. Lunde and H. Kringstad *Ibid* 32 708 (1938)

⁸⁸ H. I. C. Wilson and G. K. Roy *Indian J Med Res* 25 879 (1938)

⁸⁹ H. A. Schneider, J. L. Ascham, B. R. Platz and H. Steenbock *J Nutrition* 18 99 (1939)

⁹⁰ N. Halliday and H. M. Evans *J Biol Chem* 118 250 (1937)

⁹¹ M. K. Dimick and C. B. Schreffler *J Nutrition* 17 23 (1937)

⁹² E. J. Reedman, W. L. Sampson and K. Unn *Proc Soc Exptl Biol Med* 43 112 (1940)

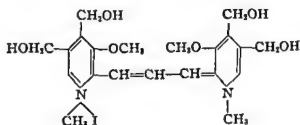
⁹³ P. György *Ibid* 35 204 (1936)

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⁷³ J. V. Scudé, H. F. Koones and J. C. Keresztesy, *Proc. Am. Physiol. Soc.* 1940, 163; *Proc. Soc. Exptl. Biol. Med.* 43, 118 (1940).

⁷⁴ J. V. Scudé, K. Unna and W. Antopol, *J. Biol. Chem.* 135, 371 (1940).

Little is known about the physiological action of vitamin B₆. It has been suggested that vitamin B₆ is connected with the utilization of unsaturated fatty acids¹⁰⁷. It has also been observed that in vitamin B₆ avitaminotic rats, the livers are significantly heavier and contain a higher percentage of total fatty acids¹⁰⁸ which increase cannot be correlated to the food intake¹⁰⁹. Rats maintained on a vitamin B₆ deficient diet can according to some investigators be protected from the symptoms of vitamin B₆ deficiency by supplementing the diet with the essential unsaturated fatty acids¹¹⁰⁻¹¹¹. Others were unable to obtain this effect but called attention to the very similar type of dermatitis in both vitamin B₆ and essential fatty acid deficiency which, however, can be differentiated since edema occurs only in the case of vitamin B₆ deficiency. The crux of the problem of the relation of vitamin B₆ to the fat metabolism is that the animal organism (tested on rats) needs vitamin B₆ for the synthesis of fat from protein¹¹². This vitamin is apparently concerned with the metabolism of the amino acids, as has also been shown in a determination of the protein and carbohydrate appetite in rats by the self selection method¹¹³.

The mechanism of the vitamin B₆ action is still unknown. Upon the discovery¹¹⁴ that vitamin B₆ occurs in tissues partly bound to proteins it was suspected that the principal function of this vitamin is to act as part of some enzyme system, like some of the other members of the vitamin B group. The result of an approach to solve this problem was negative: the methyl iodo compound of vitamin B₆ is not reduced to a dihydro compound¹¹⁵ in a manner similar to the reduction of the methyl iodo compound of nicotinamide (see page 238).

15 Avitaminosis and Hypovitaminosis

Vitamin B₆ avitaminosis in rats causes a specific symmetrical dermatitis which is called acrodynia¹¹⁶ and which affects primarily the peripheral parts of the body such as the paws, the mouth, the tail, the ears and nose¹¹⁷.

¹⁰⁷ T. W. Breh, *J. Biol. Chem.* 124, 775 (1938).

¹⁰⁸ N. Halliday, *J. Nutrition* 16, 285 (1938).

¹⁰⁹ R. W. Fingel, *Proc. Am. Soc. Biol. Chem.* 1941, XXXVII.

¹¹⁰ F. W. Quack and H. Steinhilber, *Proc. XVI Intern. Physiol. Congr. Zurich* 1938, 108.

¹¹¹ W. D. Salmon, *Proc. Am. Soc. Biol. Chem.* 34, LXXXII (1940).

¹¹² E. W. McHenry and G. Gavin, *J. Biol. Chem.* 138, 471 (1941).

¹¹³ C. P. Richter and C. D. Hawkes, *Am. J. Physiol.* 129, 459 (1940).

¹¹⁴ R. Kuhn and G. Wendt, *Ber.* 71, 780 (1938).

¹¹⁵ R. Kuhn and I. Löw, *Ibid.* 72, 1453 (1939).

¹¹⁶ T. W. Breh, P. György and I. J. Harris, *Biochem. J.* 29, 2830 (1935).

¹¹⁷ W. Jefremow, *Vopr. Fiziol. i Med. Biol.* 7, No. 3, 43 (1938). See *Chem. Zvesti* 1, 1939, 4196. *Isop. org. i med. biol.* 6, No. 1, 55 (1937). See *Chem. Zvesti* 1, 1938, 1132.

13 Physiology of Plants and Microorganisms

Although it can be assumed that vitamin B₆ is necessary for plants and microorganisms, only few actual facts are known. It has been demonstrated experimentally that vitamin B₆ is a nutrilit for yeast^{94, 95} and *staphylococcus albus*⁹⁶ and that it is necessary for lactic acid bacteria⁹⁷ and for some hemolytic streptococci.⁹⁸ Some other bacteria are able to synthesize vitamin B₆, for example, those in the rumen of sheep.⁹⁹ Only a few observations concerning the vitamin B₆ action in higher plants are available. Vitamin B₆ acts as a growth stimulant¹⁰⁰ on isolated tomato roots and cosmos.¹⁰¹ The isolated pea root does not require an external supply of vitamin B₆ and it must be presumed that the root is able to build up the vitamin from compounds present in the culture medium.¹⁰¹ Analytical determination of the vitamin B₆ content of wheat and maize plants seems to indicate a greater concentration of this vitamin in the germs and the integuments than in the endosperm.¹⁰²

14 Animal Physiology

Free vitamin B₆ is rapidly absorbed in the intestines.¹⁰³ Vitamin B₆ bound to protein material is not absorbed. Plant material, for example seeds and vegetables, should be cooked to insure full utilization of the vitamin B₆ present¹⁰⁴ (as shown in experiments with rats).

Investigations on the behavior of vitamin B₆ in the human organism are scarce. Vitamin B₆ has not been detected in blood¹⁰⁵ (a reinvestigation seems advisable) but is present in urine,^{105, 106} which indicates that this compound is of physiological importance for man. The vitamin is also secreted in milk. When excess amounts of vitamin B₆ are administered, they are largely destroyed in the organism. Storage of this vitamin in the organism has not been observed.

⁹⁴ A. S. Schultz, L. Atkin and C. N. Frey, *J. Am. Chem. Soc.* **61**, 1931 (1939).

⁹⁵ R. E. Eakin and R. J. Williams, *Ibid.* **61**, 1932 (1939).

⁹⁶ S. P. Vilter and T. D. Spies, *Science* **91**, 200 (1940).

⁹⁷ E. F. Möller, *Z. physiol. Chem.* **254**, 285 (1938).

⁹⁸ B. L. Hutchings and D. W. Woolley, *Science* **90**, 41 (1939).

⁹⁹ L. W. McElroy and H. Goss, *J. Biol. Chem.* **130**, 437 (1939).

¹⁰⁰ W. J. Robbins and M. B. Schmidt, *Proc. Natl. Acad. Sci. U. S.* **25**, 1 (1939).

¹⁰¹ J. Bonner, *Am. Chem. Soc. Div. Agr. Food Chem. Meeting*, Sept. 1939, Abst. p. 13.

¹⁰² A. M. Copp, *ing. Biochem. J.* **30**, 849 (1938).

¹⁰³ J. V. Scudis, K. Unna and W. Antopol, *J. Biol. Chem.* **135**, 371 (1940).

¹⁰⁴ E. M. Lantz, *New Mexico Agr. Exptl. Station Bull.* No. 268.

¹⁰⁵ Cited from W. Stepp, J. Kühnau and H. Schroeder, *Die Vitamine*, 2nd edit., Stuttgart, 1937, p.

tion has also been called to the possibility that chilblains may afford a clinical demonstration of vitamin B₆ deficiency¹³⁹ Promising results have also been obtained in the treatment of myasthenia^{137 140} Improvement has furthermore been noted in patients with idiopathic epilepsy¹³⁷ and with macrocytic anemia of pellagra or pernicious anemia in relapse¹⁴¹

(a) Clinical Test Methods

The Urine Test In urine the excretion of vitamin B₆ can be demonstrated for rats and for man¹⁴² The methods used are either the chemical indophenol test or the biological rat test The former can be applied to urine when it contains 1 γ per cc, but not when less material is present Urine also contains substances which interfere with the test In human beings vitamin B₆ can be demonstrated in the urine only after the intake of excessive doses The normal urinary excretion of man and dogs is less than 0.5 γ per cc, while rats excrete about 0.5–1.0 γ per cc

The Blood Test There is no method known by which vitamin B₆ can be determined in blood As a matter of fact the presence of this vitamin in blood has not been demonstrated as yet

16 Hypervitaminosis

Vitamin B₆ is a substance of relatively low toxicity^{143 144} Chronic toxicity was studied in rats, dogs and monkeys by daily feeding up to 10 mg per kilogram body weight over periods extending to three months No significant differences in weight or in the hemoglobin, erythrocytes, leucocytes, etc., were observed Twenty mg per kilogram body weight, injected intravenously into cats, had no effect Single doses up to 1 g were tolerated without untoward effects Higher doses produced tonic convulsions and suggest involvement of certain parts of the nervous system The lethal dose in rats is about 3 g per kilogram body weight¹⁴⁵ Vitamin B₆ has a sedative effect in man¹⁴⁶

¹³⁹ P György *J Nutrition* 16 61 (1938)

¹⁴⁰ J V Seud H F Koones and J C Keresztesy *Proc Am Physiol Soc* 1940 163 *Proc Soc Exptl Biol Med* 43 118 (1940)

¹⁴¹ R W Vilter H b Schiro and T D Spies *Nature* 145 388 (1940)

¹⁴² T D Spies R K Ladisch and W B Bean *J Am Med Assoc* 115 839 (1940)

¹⁴³ K Unna and W Antopol *Proc Soc Exptl Biol Med* 43 116 (1940)

¹⁴⁴ K Unna *Am J Physiol* 129 483 (1940)

¹⁴⁵ T D Spies D P Hightower and L H Hubbard *J Am Med Assoc* 115 292 (1940)

and which is accompanied by edema and scaliness¹¹⁵ Furthermore, rats cease growing^{119 120} In dogs,¹²¹ rats^{122 123 124} and pigs^{125 126} fits of an epileptiform nature were observed besides the symptoms of subnormal growth and dermatitis The animals become abnormally excited and any extra stimulus induces fits The striated and cardiac muscles degenerate, and pathological changes have been noted in the nervous system, especially in the columns of the spinal cord Chicks also need vitamin B₆,^{127 128} but no characteristic dermatitis can be observed during times of vitamin B₆ deficiency The symptoms in chicks consist of slow growth, depressed appetite and inefficient utilization of food In some cases spastic convulsions and death were observed¹²⁹ In dogs, vitamin B₆ deficiency causes a microcytic hypochromic anemia^{130 131 132 133} and in rats a thymus atrophy has been observed¹³⁴ In rats kept on a vitamin B₆ deficient diet the *accessory organs of reproduction are reduced and the animals show defective sexual behavior*¹³⁵

The present day knowledge of the action of vitamin B₆ in human beings is very limited The necessity of this vitamin for man has not been proved, but it seems reasonable to assume that vitamin B₆ is a vitamin in human nutrition Upon administration of vitamin B₆ to pellagrins, recovery has been observed in some cases in which the vitamins B₁, B₂ and nicotinic acid failed to remove the symptoms¹³⁶ These symptoms include nervousness insomnia, irritability, cramping pains in the stomach, muscular weakness and muscular rigidity¹³⁷ Patients with pseudohypertrophic muscular dystrophy, for example, respond well to vitamin B₆ treatment¹³⁸ Atten

- ¹¹⁵ W Antopol and K Unna *Proc Soc Exptl Biol Med* 42 126 (1939)
¹¹⁶ R Kuhn and G Wendt *Z physiol Chem* 256 127 (1938)
¹¹⁷ C E Edgar M M El Sadr and T F Macrae *Biochem J* 32 2207 (1938)
¹¹⁸ P J Fouts O M Helmer S Lepkovsky and T H Jukes *J Nutrition* 16 197 (1938) J M
 McKibbin R J Madden S Black and C A Elvehjem *Am J Physiol* 128 102 (1939)
¹¹⁹ H Chick A N Worden and M M El Sadr *J Soc Chem Ind* 58 1019 (1939)
¹²⁰ H Chick M M El Sadr and A N Worden *Biochem J* 34 595 (1940)
¹²¹ J J Oleson H R Bird C A Elvehjem and E B Hart *J Biol Chem* 127 23 (1939)
¹²² H Chick T F Macrae A J P Martin and C J Martin *Biochem J* 32 2207 (1938)
¹²³ M M Wintrobe *Am J Physiol* 126 375 (1939)
¹²⁴ D M Hegsted J J Oleson C A Elvehjem and E B Hart *J Biol Chem* 130 423 (1939)
¹²⁵ C W Carter and J R O'Brien *Proc 7th World's Poultry Congress and Exposition* 1939 125
¹²⁶ T H Jukes *Proc Soc Exptl Biol Med* 42 180 (1939)
¹²⁷ P J Fouts O M Helmer S Lepkovsky and T H Jukes *J Nutrition* 16 197 (1938) P J
 Fouts O M Helmer and S Lepkovsky *Proc Soc Exptl Biol Med* 40 4 (1939)
¹²⁸ H J Jukes and S R Mettler *Ibid* 43 429 (1940)
¹²⁹ G Lundie and H Kringstad *Biochem J* 32 708 (1938)
¹³⁰ H E C Wilson and G K Roy *Indian J Med Research* 25 879 (1938)
¹³¹ M K Dimick and C B Schreffler *J Nutrition* 17 23 (1939)
¹³² G A Emeron and H M Evans *Am J Physiol* 129 350 (1940)
¹³³ T D Spies W B Bean and W F Ashe *J Am Med Assoc* 112 2414 (1939)
¹³⁴ T D Spies D P Hightower and I H Hubbard *Ibid* 115 292 (1940)
¹³⁵ W Antopol and C E Schotland *Ibid* 114 1058 (1940)

NICOTINIC ACID—
NICOTINAMIDE

17 Requirements

Since vitamin B₆ has not yet been demonstrated as necessary for human life, no data are available as to the requirements of this vitamin for human beings. In a few clinical cases daily doses of 10–100 mg. were given.¹⁴⁶

The requirements for rats have been repeatedly investigated and were found to be 10 γ daily^{147, 148} of the vitamin hydrochloride. Chicks also need this vitamin (about 30 γ per day).¹⁴⁹ Sheep, however, do not require an external supply of vitamin B₆, since it is synthesized by bacteria in the rumen.¹⁵⁰ The same has also been reported for cattle.^{151, 15}

¹⁴⁶ T. D. Spies, W. B. Bean and W. F. Ashe, *J. Am. Med. Assoc.* **112**, 2414 (1933).

¹⁴⁷ R. Kuhn and G. Wendt, *Ber.* **71**, 780 (1938).

¹⁴⁸ M. K. Dimick and C. B. Schreffler, *J. Nutrition* **17**, 23 (1939).

¹⁴⁹ D. M. Hegsted, J. J. Oleson, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.* **130**, 493 (1939).

¹⁵⁰ L. W. McElroy and H. Goss, *Ibid.* **130**, 437 (1939).

¹⁵¹ L. W. McElroy and H. Goss, *Ibid.* **133**, LXV (1940).

¹⁵² M. I. Wegner, A. N. Booth, C. A. Elvehjem and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.* **45**, 769 (1940).

NICOTINIC ACID—
NICOTINAMIDE

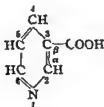
NICOTINIC ACID—NICOTINAMIDE

1 Nomenclature and Survey

Names

- Nicotinic acid and nicotinamide
- Niacin synonym for nicotinic acid
- Niacin amide synonym for nicotinamide
- P P factor Pellagra Preventive factor¹
- Pellagramine²
- Niamid Suggested abbreviation for nicotinamide

Chemical formulas



Nicotinic acid



Nicotinamide
(abbreviated formula)

Chemical names

- Pyridine 3-carboxylic acid and acid amide
- Pyridine β carboxylic acid and acid amide

Empirical formulas

- Nicotinic acid $C_6H_5O_2N$
- Nicotinamide C_6H_6ON

Nicotinic acid may possibly be identical with vitamin B₃. The term vitamin B₃ was given originally to a fuller's earth eluate fraction from yeast and was shown to be necessary for the growth of pigeons. Vitamin B₃ was later found to be present in vitamin B₆ fractions but could not fully replace the vitamin B₃ requirements of pigeons. The remaining factor designated as vitamin B₃ appears to be identical with nicotinic acid.³

¹ The term pellagra preventive factor was applied to the substance originally postulated as necessary for the prevention of human pellagra. J. Goldberger and W. F. Tanner. *U. S. Pub. Health Service Pub. Health Repts.* 39: 87 (1944).

² R. L. Jones. *Science* 68: 480 (1948).

³ C. W. Carter and J. R. O'Brien. *Biochem. J.* 33: 1810 (1949).

2 Chronology

- 1735 First description of the human disease pellagra by CASAL
- 1907 SEARCY reported the occurrence of pellagra in epidemic forms
- 1912-1914 FUNK⁴ in Europe and SUZUKI⁵ in Japan isolated nicotinic acid from yeast and rice bran during their search for the antipolyneuritic vitamin (vitamin B₁) but failed to recognize the vitamin character of the isolated compound FUNK observed however the beneficial effect of nicotinic acid when given in mixture with the antipolyneuritic vitamin
- 1917 CHITTENDEN and UNDERHILL⁶ produced a pathological condition resembling human pellagra in dogs which is called canine blacktongue
- 1926 GOLDBERGER and LILLIE obtained an experimental pellagra like condition in albino rats by feeding a diet deficient in a substance called Pellagra Preventive Factor (P P Factor)⁷
- 1928 GOLDBERGER and associates⁸ collected evidence that substances capable of curing blacktongue in dogs were equally effective in the cure of human pellagra
- 1929 AYKROYD and ROSCOE recognized that the pellagra preventive factor and the anti blacktongue factor always occur together⁹
- 1930 NORRIS and RINGROSE¹⁰ produced a pellagra like condition in chicks
- 1935 WARBURG and CHRISTIAN¹¹ and EULER ALBERS and SCHLENK¹² demonstrated that nicotinamide is a part of the hydrogen transporting coenzymes
- 1936 EULER and MALMBERG¹³ reported that nicotinic acid prolonged the lives of rats fed vitamin B₁ vitamin B₂ and vitamin B₆
- 1937 KNIGHT¹⁴ and MUELLER¹⁵ found nicotinamide to be essential to the growth of certain unicellular organisms ELVEHJEM and co-workers¹⁶ showed that nicotinic acid is effective in the cure of blacktongue in dogs and that it can be isolated from anti blacktongue active liver extracts FOUTS HELMER LEFKOVSKY and JUKES¹⁷ reported the first successful treatment of human pellagra with nicotinamide

⁴ C Funk *J Physiol* 46 173 (1913) *Brit Med J* 1913 I 814 J C Drummond and C. Funk *Biochem J* 8 594 (1914)

⁵ U Suzuki and S Matsunaga *J Agr Tokyo Imp Univ* 5 99 (1912) U Suzuki T Shammura and S Okada *Biochem Z* 43 89 99 (1912)

⁶ R H Chittenden and F P Underhill *Am J Physiol* 44 13 (1917)

⁷ J Goldberger and R D Lillie *U S Pub Health Service Pub Health Repts* 41 10 5 (1926)

⁸ J Goldberger and G A Wheeler *Ibid* 43 172 (1928) J Goldberger G A Wheeler R D Lillie and L M Rogers *Ibid* 43 1385 (1928) J Goldberger G A Wheeler L M Rogers and W H Sebrell *Ibid* 45 1297 (1930)

⁹ W R Aykroyd and M H Roscoe *Biochem J* 23 483 (1929)

¹⁰ L C Norris and A T Ringrose *Science* 71 643 (1930)

¹¹ O Warburg and W Christian *Biochem Z* 275 464 (1935) O Warburg W Christian and A Criesel *Ibid* 279 143 (1935)

¹² H v Euler H Albers and F Schlenk *Z physiol Chem* 237 1 (1935)

¹³ H v Euler and M Malmberg *Biochem Z* 284 455 (1936)

¹⁴ B C J C Knight *Biochem J* 31 731 (1937)

¹⁵ J H Mueller *J Bact* 34 429 (1937) *J Biol Chem* 120 219 (1937)

¹⁶ C A Elvehjem R J Madden F M Strong and D W Woolley *J Am Chem Soc* 59 1767 (1937) *J Biol Chem* 123 137 (1938)

¹⁷ P J Fouts O M Helmer S Lefkovsky and T H Jukes *Proc Soc Exptl Biol Med* 37 405 (1937)

3 Occurrence of Nicotinic Acid and of Nicotinamide

Nicotinic acid occurs in all living cells in small amounts. The liver¹⁸ the adrenal gland¹⁹ and yeast and wheat germs are especially rich in nicotinic acid. The eye lenses contain a fair amount¹⁹ and corn meal corn syrup alfalfa²⁰ fatty meat and milk¹ contain small amounts.

Free nicotinic acid apparently does not occur in the living organism but is found in the urine of animals. Nicotinic acid occurs in tissues in the form of its amide. Besides the occurrence of the free nicotinamide there exists a number of enzyme systems (see page 227) in which nicotinamide is chemically bound. Nicotinamide occurs to a much greater extent in the bound form than as the free nicotinamide. Thus in rats bound nicotinamide occurs for example in the liver, kidney and muscles while free nicotinic acid (or its amide) has been found only in the liver.

4 Isolation of Nicotinic Acid and of Nicotinamide

The isolation of nicotinic acid from natural sources is a relatively simple matter. Tissue material is freed from fats by extraction with organic solvents. The remaining material is saponified, preferably with alkali. The acid fraction of the saponification mass contains the nicotinic acid which can be separated as such as the ester or as the Cu salt. From this salt the free acid is obtained by means of hydrogen sulfide.

The isolation of the total nicotinamide from animals or plants is carried out by water extraction of the material followed by partial hydrolysis in 0.1 N sulfuric acid in order to split the nicotinamide from its chemical combination with the various enzymes. The water phase is then extracted with butanol or chloroform². The chloroform solution is subjected to fractional distillation. Nicotinamide distills at 150–160° C at 5×10^{-4} mm Hg³. The distillate may be recrystallized from chloroform and benzene.

The separation of free nicotinic acid from nicotinamide is effected by solvent extraction (ether chloroform butanol) of a water solution. The amide is soluble in organic solvents while the free acid remains in the water phase.

¹⁸ W. J. Dann, *Science* 86, 618 (1937).

¹⁹ E. Kodicek, *J. Soc. Chem. Ind.* 58, 1088 (1939).

²⁰ W. R. Wytt, *Iowa State Coll. J. Sci.* 14, 103 (1939).

¹ E. Kodicek, *Biochem. J.* 34, 712–724 (1940).

² H. v. Euler, P. Schlenk, L. Meitzer and B. Högberg, *Z. physiol. Chem.* 258, 21 (1939). P. Karrer and H. Krell, *Helv. Chim. Acta* 22, 1292 (1939).

³ R. Kuhn and H. Vetter, *Ber.* 68, 2374 (1935).

The separation of free nicotinamide from chemically bound nicotinamide (coenzymes) must be carried out immediately after death,^{24 25} and is usually achieved by an acetone extraction of the material. Acetone dissolves nicotinamide, but does not dissolve the coenzymes. Acetone, furthermore, prevents the naturally occurring enzyme systems from splitting the nicotinamide containing coenzymes into their components.

5 Properties of Nicotinic Acid and of Nicotinamide

1 Nicotinic acid crystallizes in needles from water or alcohol and melts at 235.5–236.6° C.⁶ It sublimes without decomposition. Nico

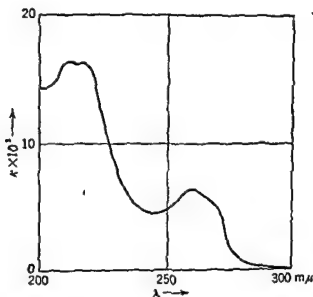


Fig. 12—Absorption spectrum of nicotinamide in water (R. Kuhn and H. Vetter)

tinic acid exhibits a typical absorption spectrum with a maximum at 385 mμ.²⁶

2 Nicotinamide crystallizes in needles from benzene and melts at 129° C. It distills at 150–160° C and 5×10^{-4} mm Hg.²⁸ The absorption spectrum of nicotinamide is shown in Fig. 12.

²⁴ H. v. Euler, F. Schlenk, L. Melzer and B. Högberg, *Z. physiol. Chem.* 258, 212 (1939).

²⁵ H. v. Euler and K. Myrbäck, *Ibid.* 117, 237 (1928); H. v. Euler and C. Günther, *Ibid.* 243, 1 (1936); H. v. Euler, H. Heiwinkel and F. Schlenk, *Ibid.* 247, IV (1937).

²⁶ R. Gording and L. A. Flexner, *J. Am. Pharm. Assoc.* 29, 230 (1940).

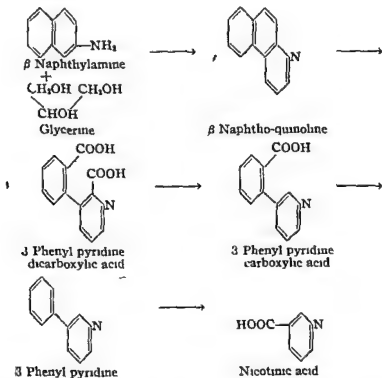
²⁷ H. Hünecke, *Ber.* 60, 1451 (1927).

²⁸ R. Kuhn and H. Vetter, *Ibid.* 68, 2374 (1935).

6 Constitution of Nicotinic Acid and of Nicotinamide

The constitution of nicotinic acid was determined when this acid was first obtained by oxidation of nicotine⁹ from which the name of this acid originates

The acid character was established by the formation of a silver and a copper salt and by the formation of various derivatives such as esters acid chloride etc. The basic character was recognized by the formation of crystallized salts such as the hydrochloride the hydrobromide etc. By distillation of the calcium salt of the acid the carboxylic acid group is split off and pyridine is obtained¹⁰



The *m* or 3 position of the carboxylic acid group in reference to the ring nitrogen was suspected by Skraup who investigated the physical constants and the decarboxylation of the three possible pyridine mono carboxylic acids: picolinic acid (*o* or 1,2) nicotinic acid (*m* or 1,3) and γ pyridine carboxylic acid (*p* or 1,4 position)¹¹. The *m* position was proved to be the correct one upon oxidation of the synthetically prepared 3-phenyl

⁹ C. Huber *Ber.* 3 849 (1870) *Ann.* 141 71 (1867) H. Wendt *Ann.* 165 346 (1873)

¹⁰ H. Wendt *Ann.* 165 331 (1873)

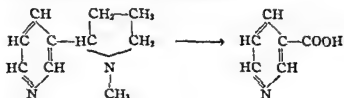
¹¹ Z. H. Skraup *Afionolish* 1 800 (1880)

pyridine the constitution of which is beyond doubt } Phenyl pyridine was prepared from β naphtho quinoline which in turn was synthesized from β naphthylamine and glycerine β Naphtho quinoline yields upon oxidation with permanganate β phenyl pyridine dicarboxylic acid, which by stepwise decarboxylation yields the mono carboxylic acid and finally 3 phenyl pyridine³² A further proof for the 3 position of the carboxylic acid group in nicotinic acid is its formation from the synthetically prepared *m* dipyridyl³³

7 Synthesis

(a) Nicotinic Acid

1 By Oxidation of Nicotine This is the method by which nicotinic acid was discovered The oxidation may be accomplished by fuming nitric acid,³⁴ by chromic acid³⁵ or by permanganate³⁶



2 By Oxidation of β -Pyridines This method is really a generalization of the method first discussed β Picoline,³⁷ 3 ethyl pyridine,³⁸ 3 phenyl pyridine,³⁹ 3,3' dipyridyl⁴⁰ and similar compounds have been converted by this method into nicotinic acid

3 By Decomposition of Pyridine-poly-carboxylic Acids Any pyridine poly carboxylic acid, which has one carboxylic acid group in 3 position can be converted into the 3 mono carboxylic acid by thermal decomposition⁴¹ or by acidic⁴ decomposition An exception to this rule

³² Z H Skraup and A Cobenzl *Monatsh* 4 436 (1883)

³³ Z H Skraup and G Vortmann *Ibid* 4 594 (1883)

³⁴ H Weidel *Ann* 165 331 (1873)

³⁵ C Huber *Ber* 3 849 (1870) *Ann* 141 271 (1867) H Weidel *Ann* 165 346 (1873)

³⁶ R Laiblin *Ber* 10 2131 (1877) *Ann* 196 130 (1879)

³⁷ H Weidel *Ber* 12 1932 2004 (1879) H Ost *J prakt Chem* [2] 27 286 (1883) J Seyffert *Ibid* [2] 34 258 (1886)

³⁸ H Weidel and K Hazura *Monatsh* 3 783 (1882) A Iadenburg *Ann* 301 162 (1898) A Wischnegradski *Ber* 12 1480 (1879)

³⁹ Z H Skraup and A Cobenzl *Monatsh* 4 458 (1883)

⁴⁰ Z H Skraup and G Vortmann *Ibid* 4 594 (1883)

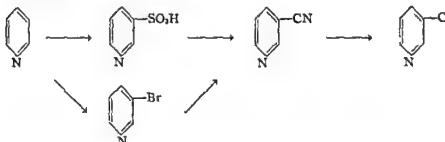
⁴¹ S Hoogewerff and W A van Dorp *Ann* 204 117 (1880) *Ibid* 207 219 226 (1881) *Rec trav chim* 1 122 (1882) R Camps *Arch Pharm* 240 353 359 (1902) J Weidel and J Herzog *Monatsh* 1 16 (1880) F B Ahrens and R Gorkow *Ber* 37 2063 (1904)

⁴² H Ost *J prakt Chem* [2] 27 286 (1883) S Hoogewerff and W A van Dorp *Ber* 14 914 (1881) H Weidel and J Herzog *Monatsh* 6 982 (1885)

PREPARING NICOTINIC ACID AND NICOTINAMIDE

is the 3,4 dicarboxylic acid which decarboxylates only under very conditions yielding pyridine 4 carboxylic acid

4 **By Synthesis from Pyridine** This is a total synthesis of nicotinic acid. Pyridine is sulfonated with fuming sulfuric acid, yielding sulfonic acid. By distillation of its sodium salt with potassium cyanide, nicotinic acid nitrile is obtained.⁴³



Considerably better yields are obtained by first brominating pyridine at the 3 position followed by conversion into 3 cyano pyridine by means of cuprous cyanide.⁴⁴ Saponification of the nicotinonitrile yields nicotinic acid.

(b) *Nicotinamide*

1 By Amidation of Nicotinic Acid Nicotinamide is obtained by passing ammonia gas into nicotinic acid at 230°C "

2 By Amidation of Esters of Nicotinic Acid The methyl or ester of nicotinic acid yields nicotinamide on reaction with aque better, alcoholic ammonia⁴⁶

8 Industrial Methods of Preparing Nicotinic Acid and Nicotin

Nicotinic acid and its amide are prepared according to the method described before. The nitric acid oxidation of nicotine, a relatively alkaloid obtained as a by product from tobacco, is of special interest. Alternative procedures are permanganate oxidations of β -picotinic acid. For technical purposes the oxidation of the fraction of nicotine from coal tar or petroleum which distills between 135° and 142° C. has been reported. The oxidation of the corresponding fraction of bone tar oil⁴⁹ has been considered.

© Fischer B r 15 63 (188)

S. M. McElvain and M. A. Goese *J. Am. Chem. Soc.* **63**, 283 (1941).

⁴ S. K. Matsuo, K. Yokota, and I. Satoda, *J. Pharm. Soc. Japan* 53, 994 (1933).

* C Engler Ber 27 1787 (1894) R Camps Arch Pharm 240 354 (1907) F Po
netch 16 53 (1895) F B La Forge J Am Chem Soc 50 2477 (1928)

¹ *Org. Syntheses Coll. Vol. 1* 378 (1932).

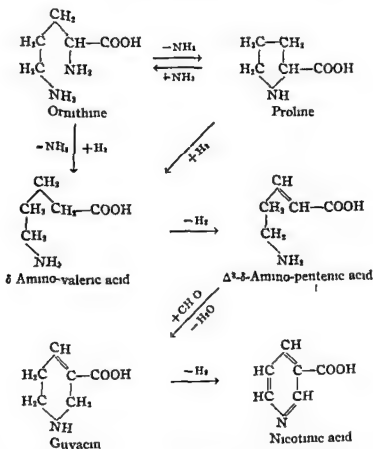
* A. Finner *Ber* 33 1227 (1900)

* H. Wendel *Ibid.* 12 1992 (1879).

A number of *N* substituted nicotinamide derivatives have attracted commercial interest because of their clinical use in the treatment of shock, collapse and cardiac decompensation⁵⁰ The most useful compound of these derivatives is the *N* diethyl nicotinamide

9 Biogenesis of Nicotinic Acid

The biogenesis of nicotinic acid is not known with certainty, but is believed to be linked with the amino acid metabolism. It has been suggested⁵¹ that nicotinic acid may originate from ornithine or proline. The first reaction product would be δ amino valeric acid. This reaction is common for bacteria⁵² but has not been proved for plants. The further course of the suggested nicotinic acid synthesis is indicated in the following scheme



⁵⁰ A selected number of patents for the preparation and use of these compounds appear in the patent index at the end of the book

⁵¹ M. Guggenheim *Die biogenen Amine* New York 1940 p. 174

⁵² D. Ackermann *Z. Biol.* 57, 104 (1910); C. Neuberg *Biochem. Z.* 37, 490 (1911); E. Salkowski and H. Salkowski *Ber.* 16, 1191 (1883)

10 Enzyme Systems Containing Nicotinamide

All plant and animal cells contain among their enzyme systems c dehydrogenases which transport hydrogen and take care of a numl different dehydrogenation reactions. The classical conception is each of these enzymes or, better, holoenzymes consists of an apoen and a coenzyme. The apoenzyme, which is believed to have no cat properties of its own, is the protein bearer of the coenzyme, which garded as the prosthetic group of the protein.⁵³ Another view is th protein is the enzyme itself and that the coenzyme merely acts as a substrate to accept hydrogen.⁵⁴ There are two coenzymes known o class of dehydrogenases, namely codehydrogenases I and II, whic also called coenzymes I and II. The number of apoenzymes combine with these two coenzymes is considerably greater. It is bel that the two coenzymes need different apoenzymes for their acti

TABLE I

Substrate system	Apoenzyme source	Coen
β Hydroxy butyrate \rightleftharpoons acetoacetate ⁵⁵	Cardiac muscle	1
Formate \rightarrow CO ₂ + H ₂ O ⁵⁶	Dried pea seeds	1
Lactate \rightleftharpoons pyruvate ⁵⁷	Skeletal muscle	1
Malate \rightleftharpoons oxaloacetate ⁵⁷	Skeletal muscle	1
Alcohol \rightleftharpoons acetaldehyde	Yeast ^{58, 59, 60}	1
	Liver ⁶¹	1
Glucose \rightarrow gluconic acid	Liver ⁶²	10
Glutamic acid \rightleftharpoons α keto glutaric acid + NH ₃ ^{63, 64}	Plants	1
	Yeast	1
	Liver	10
	Liver	1
2 Aldehydes \rightarrow 1 alcohol + 1 acid ⁷ (aldehyde mutation)		
α Glycerophosphate \rightarrow phosphoglycerate ⁶⁵	Skeletal intestinal and cardiac muscle	1
Phospho glyceraldehyde \rightleftharpoons diphospho glycerate ⁶⁶ (triose catabolism) ⁷⁰	Skeletal and cardiac muscle brain	1
Glucose 6 phosphate \rightarrow 6 phospho gluconate ¹	Yeast erythrocytes	1
6 Phospho-gluconate \rightarrow phospho keto hexonate ⁷¹	Yeast animal tissues	1
Citrate \rightarrow α keto-glutarate ⁷²	Liver heart	1

(See following page for table footnotes)

⁵³ O Warburg, *Lehrbuch Enzymforschung* 7-10 (1937)⁵⁴ M Dixon and L. G. Zervas, *Biochem J* 34: 371 (1940)

oxidizing and as reducing agents. Specific proteins are used for each substrate and a specific protein may in special cases dehydrogenate the same substrate with different coenzymes.

Table I summarizes the better known dehydrogenation reactions in which the codehydrogenases participate. It is evident that these coenzymes are involved in a wide variety of reactions. There are quite probably other such reactions, which have not been thoroughly investigated. It has, for example, been postulated that the oxidation of cysteine to cystine involves the participation of a codehydrogenase.⁷⁴

During the course of the dehydrogenation reactions indicated in the table, the coenzymes are reduced to dihydro compounds. The reverse reaction—the oxidation of the dihydro codehydrogenases to the codehydrogenases, is carried out in the presence of different apoenzymes as previously stated. Principally all dehydrogenation reactions are reversible, although in living tissues usually no such equilibrium occurs due to the fact that the reaction products do not accumulate but undergo further reaction. Practically, the equilibrium can be demonstrated in many cases such as in the system involving alcohol and acetaldehyde and is indicated in the table above for those systems for which the reversible reaction has been experimentally demonstrated. Equilibrium constants have been determined for the coenzyme I and for many of the reactions

⁷⁴ D. B. Green, J. G. Dewan and L. F. Elor, *Biochem. J.* **31**, 334 (1937); D. B. Green and J. G. Dewan, *Ibid.* **31**, 1069, 1074 (1937).

⁷⁵ E. Adler and M. Sreenivasaya, *Z. physiol. Chem.* **249**, 24 (1937).

⁷⁶ D. B. Green, D. M. Needham and J. C. Dewan, *Biochem. J.* **31**, 2327 (1937); D. B. Green and J. Brosteaux, *Ibid.* **30**, 1489 (1936); D. B. Green, *Ibid.* **30**, 2090 (1936).

⁷⁷ E. Negelein and H. J. Wulff, *Biochem. Z.* **289**, 436 (1937); **293**, 351 (1937).

⁷⁸ O. Warburg and W. Christian, *Helv. Chim. Acta* **19**, 79 (1936).

⁷⁹ H. v. Euler, E. Adler and H. Hellström, *Z. physiol. Chem.* **241**, 239 (1936).

⁸⁰ C. Lutwak Mann, *Biochem. J.* **32**, 1364 (1938).

⁸¹ D. C. Harrison, *Ibid.* **25**, 1016 (1931); E. Adler and H. v. Euler, *Z. physiol. Chem.* **232**, 6 (1933).

⁸² N. B. Das, *Z. physiol. Chem.* **238**, 269 (1936).

⁸³ H. v. Euler, E. Adler, G. Günther and N. B. Das, *Ibid.* **259**, 61 (1938).

⁸⁴ E. Adler, N. B. Das and H. v. Euler, *Sv. Vet. Akad. Ark. Kemt.* **12**, 1 (1937); J. G. Dewan, *Biochem. J.* **32**, 1378 (1938); H. A. Krebs and P. P. Cohen, *Ibid.* **33**, 1890 (1939).

⁸⁵ H. v. Euler, E. Adler and T. S. Friksen, *Z. physiol. Chem.* **248**, 227 (1937); E. Adler, G. Günther and J. E. Everett, *Ibid.* **255**, 27 (1938).

⁸⁶ M. Dixon and C. Lutwak Mann, *Biochem. J.* **31**, 1347 (1937); M. Dixon, *Ergeb. Enzymforsch.* **8**, 217 (1939).

⁸⁷ H. v. Euler, E. Adler and G. Günther, *Z. physiol. Chem.* **249**, 1 (1937).

⁸⁸ O. Warburg and W. Christian, *Biochem. Z.* **301**, 221 (1939); **303**, 40 (1939).

⁸⁹ D. B. Green, D. M. Needham and J. D. Dewan, *Biochem. J.* **31**, 2327 (1937).

⁹⁰ O. Warburg and W. Christian, *Biochem. Z.* **242**, 206 (1931); **254**, 438 (1932); F. Negelein and W. Gerischer, *Ibid.* **284**, 289 (1936).

⁹¹ F. Dickens, *Biochem. J.* **32**, 1626 (1938); F. Ismann, *Nature* **138**, 588 (1937); O. Warburg and W. Christian, *Biochem. Z.* **287**, 440 (1936); **292**, 287 (1936).

⁹² E. Adler, H. v. Euler, G. Günther and M. Plass, *Biochem. J.* **33**, 1028 (1939).

⁹³ E. Maschmann, *Naturwissenschaften* **27**, 628 (1939).

catalyzed by the coenzymes and are usually expressed in the form of the oxidation reduction potential⁷⁵. The potential E_0' for the coenzyme I at 30° C is approximately -0.29 volts⁷⁶.

In tissues the oxidation of the dihydro coenzymes may be accomplished by coenzyme linked reactions⁷⁷. It is for example possible that β hydroxy butyrate is oxidized to acetoacetate (see Table I) with the formation of dihydro cozymase I which in turn reduces an aldehyde to an alcohol. Thus the following compounds have been shown to act as acceptors in the presence of the corresponding apoenzymes: acetaldehyde, pyruvate, oxaloacetate, triose phosphate, imino glutarate (or α keto glutarate + NH_3) and fumarate⁷⁷ (in the presence of succinic dehydrogenase). It has also been shown that the riboflavin containing enzyme systems (see page 171) may be linked with the oxidation of the dihydro codehydrogenases.

11 Coenzymes Containing Nicotinamide

(a) Codehydrogenase I

Synonyms Codehydrogenase I, Coenzyme I, Cozymase, Coferment I, Diphosphopyridine nucleotide, Coferment of fermentation, Coreductase, Factor V^{78, 79, 80}.

Occurrence Codehydrogenase I has been found in all animal and plant cells in which carbohydrates are metabolized. Yeast and red blood cells are especially rich sources and some muscles for example, heart muscles contain relatively high amounts. In fresh yeast about 0.5 g of codehydrogenase I is present per kilogram⁸¹ and in the heart muscle of rabbits 0.4 g per kilogram⁸. The same amount (0.1-0.4 g) has been calculated to be present in muscles of man⁸² and of invertebrata⁸⁴. Codehydrogenase I occurs also in microorganisms and has for example, been obtained from *Azotobacter chroococcum*⁸⁵. There seems to be a fairly con-

⁷⁸ W. M. Clark, *The Determination of Hydrogen Ion Concentration*, Baltimore, 1928. I. Michaeli, *Oxidation Reduction Potentials*, Berlin, 1933.

⁷⁹ F. Schlenk, H. Hellström and H. v. Euler, *Ber.* 71, 1471 (1938). H. Boersook, *J. Biol. Chem.* 133, 629 (1940).

⁸⁰ J. G. Dewan and D. R. Green, *Biochem. J.* 31, 1074 (1937).

⁸¹ A. Lwoff and M. Lwoff, *Proc. Roy. Soc. (London)* B122, 359, 360 (1937). *Compt. rend.* 203, 520 (1936).

⁸² H. I. Kohn, *Biochem. J.* 32, 2075 (1938).

⁸³ T. M. Rivers, *Bull. Johns Hopkins Hosp.* 33, 149, 429 (1922).

⁸⁴ O. Meyerhof and P. Ohlmeyer, *Biochem. Z.* 290, 334 (1937).

⁸⁵ H. v. Euler, *Angew. Chem.* 50, 831 (1937).

⁸⁶ H. v. Euler, *10th Cong. Intern. Chem.* 1, 178 (1938).

⁸⁷ S. Ochoa and C. G. Ochoa, *Nature* 140, 1097 (1937).

⁸⁸ R. Nilsson, *Arch. Mikrobiol.* 7, 598 (1937).

stant ratio of coenzyme to dihydro coenzyme in the muscles of all animals, the reduced form being present in about 35–45% of the total amount⁸⁴ An increased amount of the reduced form has been found in Jensen sarcoma⁸⁵

Isolation⁸⁸ The isolation of codehydrogenase I is carried out by water extraction of the source, for example, of yeast or red blood cells It is necessary to destroy some of the other enzymes present prior to the extraction, by short heating to about 80° C, since otherwise the codehydrogenase is rapidly destroyed After filtration or dialysis some of the protein impurities are removed by precipitation with lead acetate The coenzyme itself may be extracted with phenol⁸⁹ and is precipitated by mercuric acetate or nitrate picric acid, phosphotungstic or silicotungstic acid (and decomposed by ether amyl alcohol sulfuric acid), by silver salts either in ammoniacal solution or in a solution containing barium hydroxide (and freed from silver by hydrogen sulfide), by cuprous halides in the presence of hydrochloric acid (and freed from copper by hydrogen sulfide), by ethyl acetate from an acidified methanol solution, by acetone, and by alcohol The latter is also used for fractional precipitation of this coenzyme Purification may also be accomplished by fractional adsorption on aluminum oxide or charcoal from weakly acid solutions

The methods used for the separation of nicotinamide from the coenzymes have been described in the section on the isolation of nicotinamide (page 222) The methods used for the separation of codehydrogenase I from codehydrogenase II will be found in the section on the isolation of codehydrogenase II (page 235) To separate the flavin adenine dinucleotides (see page 179) which occur together with the codehydrogenases I and II in the phenol extract from certain sources such as yeast, the flavin compounds are precipitated in acid solution as silver salts⁸⁹

Properties Codehydrogenase I is a colorless, water soluble substance, and is insoluble in most organic solvents It exhibits a characteristic absorption spectrum with a maximum at 260 mμ⁹¹ Hydrogenation to the dihydro codehydrogenase, which occurs during the enzyme action (see page 238), changes the absorption spectrum characteristically with the

⁸⁴ H v Euler F Schlenk H Heiwinkel and B Högberg *Z physiol Chem* 256 208 (1938)

⁸⁵ H v Euler M Malmberg and G Günther *Z Krebsforsch* 45 425 (1937)

⁸⁸ H v Euler *Ergeb Physiol* 38 1 (1936) O Warburg and W Christian *Biochem Z* 287 291 (1936) O Meyerhof *Ergeb Physiol* 39 10 (1937) H v Euler H Albers and F Schlenk *Z physiol Chem* 260 113 (1936) *Fortschr Chem org Naturstoffe* 1 99 (1938) P Ohlmeyer *Biochem Z* 297 66 (1938) H v Euler and E Adler *Z physiol Chem* 238, 233 (1936) B J Jandorf *J Biol Chem* 138 305 (1941) K Myrback and H Larsson *Z physiol Chem* 225 131 (1934)

⁸⁹ O Warburg and W Christian *Biochem Z* 298 150 377 (1938)

⁹⁰ J R Klein *J Biol Chem* 134 43 (1940)

⁹¹ O Warburg and W Christian *Helv Chim Acta* 19 E 79 (1936) H v Euler E Adler and H Hellström *Z physiol Chem* 241 239 (1936) K Myrback H v Euler and H Hellström *ibid* 212 7 (1932)

appearance of an additional band at 320–360 $m\mu$ with a maximum at 340 $m\mu$ (see Fig 13)

While the codehydrogenase I shows no fluorescence the dihydro compound exhibits a strong whitish fluorescence upon irradiation with ultra violet light

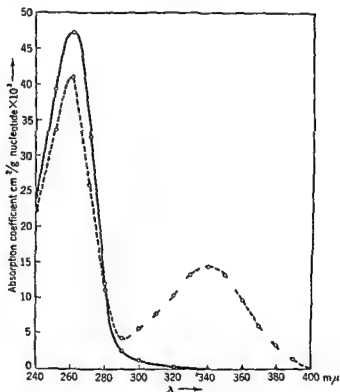


Fig 13—Absorption spectrum of codehydrogenase II in oxidized (—) and in reduced (---) form (O Warburg and W Christian)

Codehydrogenase I is optically active the specific rotation being approximately -20° for the red cadmium line ($643.9\ m\mu$)⁹ and -70° for the green mercury line ($546\ m\mu$)

Codehydrogenase I is quite stable in acid solution at moderate temperatures¹¹ whereas the dihydro codehydrogenase is destroyed by acids. In alkaline solution codehydrogenase is rapidly destroyed¹⁴ whereas the dihydro codehydrogenase remains unchanged when heated for 30 minutes

⁹ K. Myrback, H. v. Euler and H. Hellström, *Z. physiol. Chem.* 212, 7 (1932)

¹¹ K. Myrback, *Ibid.* 234, 759 (1935)

¹⁴ K. Myrback and B. Österblad, *Ibid.* 234, 254 (1935)

of codehydrogenase is the determination of the total hydrogen consumption upon catalytic hydrogenation in the presence of sodium borate

Determination by Biochemical Methods Codehydrogenase I and its dihydro form are usually determined by the degree of activation which they exert on fermentation^{114 115 116} in the presence of an excess of the apodehydrogenase. The criterion is the amount of CO₂ evolved under specified conditions which is proportional to the codehydrogenase I concentration. This method is somewhat unreliable due to the fact that the purity of the apoenzyme is not standardized¹¹⁷. This method is specific for codehydrogenase I and codehydrogenase II. Nicotinic acid and its amide do not respond to this test.

Another biochemical assay procedure for codehydrogenase I is based upon the fact that the oxidation of lactic acid by animal tissues requires the presence of codehydrogenase I¹¹⁸.

Determination by Biological Methods *Bacillus influenzae* can be used to measure accurately the total content of codehydrogenases in blood and of yeast (and probably of other sources) since this bacillus cannot synthesize the codehydrogenases from their constituents but needs the coenzymes for proper development^{119 120 121}.

Standard One unit of codehydrogenase I is defined as that quantity which produces 1 cc. of carbon dioxide in a normal fermentation¹² under specified conditions.

(b) Codehydrogenase II

Synonyms Triphosphopyridine nucleotide Warburg's Coferment, Respiratory coenzyme, Growth factor V^{122 124 125}

Occurrence Codehydrogenase II seems, like codehydrogenase I, to occur in practically all living cells. These apparently have the power

¹¹⁴ K. Myrbäck *Z. physiol. Chem.* 177 158 (1928)

¹¹⁵ A. E. Axelrod and C. A. Elvehjem *J. Biol. Chem.* 131 77 (1939)

¹¹⁶ B. J. Jandorf, F. W. Klemperer and A. B. Hastings *Ibid.* 138 311 (1941)

¹¹⁷ H. v. Euler and K. Myrbäck *Z. physiol. Chem.* 190 93 (1930)

¹¹⁸ D. E. Green and J. Brosteaux *Biochem. J.* 30 1489 (1936)

¹¹⁹ A. Lwoff and M. Lwoff *Proc. Roy. Soc. (London)* B122 352 360 (1937) *Compt. rend.* 203 520 (1936)

¹²⁰ R. W. Vilter, S. P. Vilter and T. D. Spies *J. Am. Med. Assoc.* 112 420 (1939)

¹²¹ H. I. Kohn *Biochem. J.* 32 2075 (1938)

¹²² H. v. Euler, H. Albers and F. Schlenk *Z. physiol. Chem.* 240 113 (1936). H. v. Euler and K. Myrbäck *Ibid.* 136 108 (1924). H. v. Euler and S. Karlson *Ibid.* 123 93 (1929)

¹²³ T. M. Rivers *Bull. Johns Hopkins Hosp.* 33 149 429 (1922)

¹²⁴ H. I. Kohn *Biochem. J.* 32 2075 (1938)

¹²⁵ A. Lwoff and M. Lwoff *Proc. Roy. Soc. (London)* B122 352 360 (1937) *Compt. rend.* 203 520 (1936)

of synthesizing both codehydrogenases (see under Synthesis, page 233) from nicotinic acid. It has also been postulated that the living cell is able to convert codehydrogenase I into codehydrogenase II. It seems plausible, therefore, that both coenzymes are found together. It is noteworthy that the ratio of the amounts of the two coenzymes may vary considerably in different sources. While yeast contains very little codehydrogenase II, animal tissue contains as much as 40–80 γ per gram¹⁴.

Isolation The isolation of codehydrogenase II is carried out, for example, from washed red blood cells, by destruction of the cell structure followed by a combination of various precipitation reactions^{127, 128}. Codehydrogenase II is precipitated from a water solution by acetone, by ethyl acetate especially from a methanol HCl solution by mercuric acetate by barium salts by lead salts etc.

Separation of Codehydrogenase I from Codehydrogenase II The following methods have been recommended for the separation of the two coenzymes:

1 Codehydrogenase I is separated as the cuprous salt¹²⁹ whereby codehydrogenase II remains in solution and can be isolated separately.

2 Codehydrogenase II is precipitated by lead acetate; codehydrogenase I is not¹³⁰.

3 Codehydrogenase II is more strongly adsorbed on aluminum oxide than codehydrogenase I¹²⁹. The codehydrogenase II is eluted from the Al_2O_3 by KH_2PO_4 solutions.

4 The barium salts of the two codehydrogenases can be separated by fractional precipitation with alcohol¹³¹.

Properties The properties of codehydrogenase II resemble very closely those of codehydrogenase I. They are colorless, water-soluble compounds which are insoluble in organic solvents. Codehydrogenase II is soluble in organic solvents in the presence of hydrochloric acid, for example, in methanol HCl.

Codehydrogenase II exhibits the same characteristic absorption band at 260 $m\mu$ as does codehydrogenase I. The maximum of the typical dihydro codehydrogenase absorption is at 340 $m\mu$ ¹³¹ (see Fig. 13). The latter compound also shows the characteristic fluorescence when irradiated

¹²⁸ H. v. Euler, F. Schlenk, H. Heiweck and B. Högberg, *Z. physiol. Chem.* 256, 208 (1938).

¹²⁹ F. Schlenk, *Tab. Biol.* 14, 186 (1937).

¹³⁰ O. Warburg, W. Christian and A. Griese, *Biochem. Z.* 282, 157 (1935).

¹³¹ H. v. Euler and E. Adler, *Z. physiol. Chem.* 238, 233 (1936).

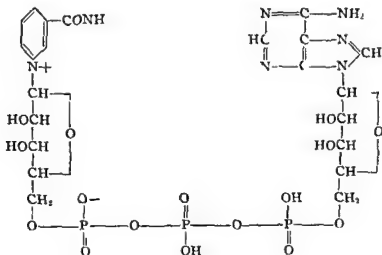
¹³² H. v. Euler, *Ibid.* 240, 113 (1936).

¹³³ O. Warburg and W. Christian, *Biochem. Z.* 287, 291 (1936).

with ultraviolet light¹³ This destroys both codehydrogenases rapidly^{133 134} Codehydrogenase II is unstable in alkaline solution, but stable in acid solution¹³⁵

In isolated muscle tissue codehydrogenase II is rapidly inactivated¹³⁶ Codehydrogenase II is optically active $[\alpha]_{589 \text{ m}\mu} = -24.6^\circ$, $[\alpha]_{546 \text{ m}\mu} = -29.4^\circ$

Constitution Codehydrogenase II has not yet been isolated in the pure form The probable empirical formula is $\text{C}_{21}\text{H}_{18}\text{N}_7\text{O}_{17}\text{P}_3$ ¹³⁷ This corresponds to 1 mol adenine, 1 mol nicotinamide, 2 mols pentose (probably *d* ribose) and 3 mols phosphoric acid Codehydrogenase II seems thus to differ from codehydrogenase I only by one additional phosphoric acid group Adenine and nicotinamide have been isolated from the breakdown products of this coenzyme^{138 139} Codehydrogenase II is dibasic since the results of electrophoresis determinations established two different dissociation constants $p_{K_1} = 1.8$ and $p_{K_2} = 6.1$ The following formula for codehydrogenase II has tentatively been suggested¹⁴⁰



¹³ O. Warburg and W. Christian *Helv. Chim. Acta* 19, 77 (1936). H. v. Euler, E. Adler and H. Hellström *Z. physiol. Chem.* 241, 239 (1936). L. Myrbäck, H. v. Euler and H. Hellström *Ibid.* 212 (1932).

¹³³ O. Warburg and W. Christian *Biochem. Z.* 282, 221 (1936).

¹³⁴ J. Runnström and L. Michaelis *J. Gen. Physiol.* 18, 717 (1935).

¹³⁵ O. Warburg, W. Christian and A. Griese *Biochem. Z.* 282, 157 (1935).

¹³⁶ H. v. Euler, H. Heiwinkel and F. Schlenk *Z. physiol. Chem.* 247, 14 (1937).

¹³⁷ O. Warburg, W. Christian and A. Griese *Biochem. Z.* 282, 157 (1935).

¹³⁸ O. Warburg and W. Christian *Helv. Chim. Acta* 19, 79 (1936). H. v. Euler, E. Adler and H. Hellström *Z. physiol. Chem.* 241, 239 (1936). L. Myrbäck, H. v. Euler and H. Hellström *Ibid.* 212 (1932).

¹³⁹ O. Warburg and W. Christian *Biochem. Z.* 275, 464 (1933).

¹⁴⁰ H. v. Euler and F. Schlenk *Z. physiol. Chem.* 246, 61 (1936).

No definite proof for this structure has as yet been obtained. As a matter of fact this structure seems doubtful in view of the fact that an apparent synthesis of codehydrogenase II from codehydrogenase I by two different methods has been accomplished (for details see below). Since these synthetic methods consist in the addition of one mol of phosphoric acid to codehydrogenase I, it would appear that the proposed formula for the coenzyme II containing three phosphoric acid groups in one chain is rather improbable. It may be that one of the phosphoric acid radicals is attached to the molecule of codehydrogenase I as a side chain, thus forming the molecule of codehydrogenase II. It is noteworthy that codehydrogenase II has apparently no free amino group since it does not react with nitrite.¹⁴¹

Synthesis It has already been pointed out in the section on Synthesis of Codehydrogenase I (p. 233) that a partial synthesis of codehydrogenase II can be accomplished from nicotinic acid or nicotinamide by the action of nucleated cells *in vitro*.

Codehydrogenase II can apparently be synthesized from codehydrogenase I since the product obtained shows the same properties as does codehydrogenase II in the test for dehydrogenating Robison ester. Ultimate proof for the accomplished conversion is still lacking. This assumed conversion of codehydrogenase I into codehydrogenase II has been carried out (1) by means of phosphorus oxychloride in ether,¹⁴² (2) by enzymatic phosphorylation.¹⁴³

Determination by Physical and Chemical Methods The same methods which were described for the determination of codehydrogenase I can also be applied to codehydrogenase II. There is no physical or chemical method of differentiating between these two coenzymes.

Determination by Biochemical Methods 1. Codehydrogenase II and its dihydro form are usually determined according to the Warburg technic by comparison with a standard preparation of this coenzyme in a system which dehydrogenates hexose monophosphoric acid (Robison ester). In addition to the coenzyme, the specific apoenzyme and the yellow ferment are also necessary.

Determination by Biological Methods Codehydrogenase II can be determined by the growth test with *Haemophilus parainfluenzae* as described for the determination of codehydrogenase I.

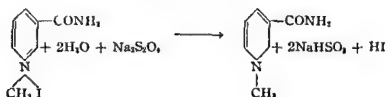
¹⁴¹ O. Warburg, W. Christen and A. Griese, *Biochem. Z.* **282**, 157 (1935).

¹⁴² F. Schlenk, *Naturwissenschaften* **25**, 668 (1937).

¹⁴³ R. V. Stanton, *J. Biol. Chem.* **25**, 667 (1937); H. v. Euler and R. Veit, *Arch. f. Chem. Mineral. Geol.* **B12**, No. 44 (1938); H. v. Euler and E. Bauer, *Ber.* **71**, 411 (1938); H. v. Euler and E. Adler, *Z. physiol. Chem.* **252**, 41 (1938).

12 Mechanism of the Nicotinamide Coenzyme Action

It has previously been stated that the action of the various nicotinamide containing enzyme systems consists in the dehydrogenation of various substrates. During this reaction, the codehydrogenase absorbs two atoms of hydrogen, thus being reduced to a dihydro form. The dihydro codehydrogenase is in turn oxidized, and thus reconverted into the codehydrogenase. The nicotinamide part of the molecule is responsible for the phenomenon of the reversible reduction-oxidation reaction of the codehydrogenases.¹⁴⁴ *In vitro* this reduction to the dihydro codehydrogenases can be brought about by the action of sodium hydrosulfite, $\text{Na}_2\text{S}_2\text{O}_4$, in weakly alkaline solution. In order to study the mechanism of this reaction and the constitution of the reduced and oxidized forms of the coenzymes, the reversible reduction of a number of simple nicotinamide derivatives has been examined.¹⁴⁵ It was found that only those derivatives which have a pentavalent ring nitrogen yield dihydro compounds with the same characteristic absorption spectrum as the dihydro coenzyme. Upon reduction, the nitrogen becomes trivalent. Nicotinamide iodomethylate proved to be the most characteristic model substance.

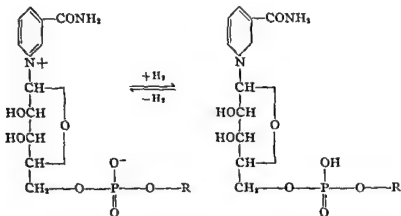


The dihydro nicotinamide compounds, but not the corresponding unreduced molecules, exhibit a typical whitish fluorescence upon irradiation with ultraviolet light. The dihydro compounds show an additional typical absorption band in the ultraviolet with a maximum at $340\text{ m}\mu$. By comparison of these characteristics with known dihydro pyridine compounds, it has been concluded that the constitution of the dihydro compound is that of an *o*-dihydro compound. Additional evidence is furnished by the deep yellow color of the dihydro compound, which would be improbable for a *p*-dihydro compound. The results of these experiments indicate that in the natural codehydrogenases the ring nitrogen of nicotinamide is chemically bound in such a way that upon reduction a tertiary

¹⁴⁴ O. Warburg, W. Christian, and A. Grise, *Biochem. Z.* **282**, 157 (1935).

¹⁴⁵ P. Karrer and O. Warburg, *Ibid.* **225**, 297 (1936); P. Karrer, G. Schwarzenbach, F. Benz, and U. V. Solmsen, *Helv. Chim. Acta* **19**, 811 (1936); P. Karrer and F. Benz, *Ibid.* **19**, 1028 (1936); P. Karrer, B. H. Ringier, J. Buchs, H. Fritzsche, and U. V. Solmsen, *Ibid.* **20**, 55 (1937); P. Karrer, G. Schwarzenbach, and G. E. Utzinger, *Ibid.* **20**, 72 (1937).

nitrogen is formed. The formula of the cohydrogenases is therefore written in the form of a quaternary pyridinium salt which in the dihydro form possesses an additional acidic group



The exact position of the reduced double bond in relation to the carboxylic acid amide group in the dihydro nicotinamide is still unknown. It is generally assumed that the double bond in $\alpha\beta$ position is reduced; the $\gamma\delta$ position however cannot be excluded on the basis of the present knowledge.¹⁴⁶

13 Specificity of Nicotinic Acid and Nicotinamide

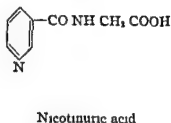
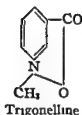
The compounds of this group which are known to be effective when given orally are nicotinic acid, its salts, for example the sodium salt, nicotinamide, the *N*-methyl amide and the *N*-diethyl amide of nicotinic acid, ethyl nicotinate, nicotinamide glucoside iodide and nicotinuric acid.¹⁴⁸ It is assumed, as would be expected on a chemical basis, that all these compounds are converted in the organism into nicotinamide. Since a number of other *N*-substituted nicotinamides have been found quite effective in the treatment of heart weakness, it is suspected that these derivatives will also prove effective as vitamins.

The ring nitrogen apparently must be unsubstituted for the exhibition of vitamin activity, since trigonelline, the nicotinic acid methyl betaine, is inactive.¹⁴⁷ This is easily understood, since in enzyme systems the ring nitrogen is attached to the rest of the enzyme molecule.

¹⁴⁶ P. Karrer, F. W. K. Bateman, R. Epstein, W. J. H. and T. Ishii, *Helv. Chim. Acta* 21, 223 (1938).

¹⁴⁷ D. W. Woolley, F. M. Strong, R. J. Madden and C. A. Elvehjem, *J. Biol. Chem.* 124, 715 (1938).

¹⁴⁸ R. W. Vilter and T. D. Spies, *Leuc.* 11, 423 (1939).



Quinolinic acid,¹⁴⁸ pyrazine carboxylic acid and pyrazine 2,3 dicarboxylic acid are active in curing human pellagra¹⁴⁹ ¹⁵⁰ (500–800 mg daily *per os*) and are said not to produce the vasodilator symptoms which often follow the administration of nicotinic acid. Dysentery bacilli can use thiazole 5 carboxylic acid as a substitute for nicotinic acid.¹⁵¹ All these compounds were found inactive in dogs.¹⁴⁷ ¹⁵²

14 Determination of Nicotinic Acid and Nicotinamide

(a) Chemical Methods

1 The Cyanogen Bromide Method This method is based on the principle¹⁵³ that pyridine derivatives give specific colors with cyanogen bromide and a primary or secondary amine. In order to determine the quantity of nicotinic acid in natural products, the latter must be totally hydrolyzed to contain only the free acid. The solutions, protected from light, are warmed with CNBr in the dark and treated with an amine¹⁵⁴ for example, with *p* amino acetophenone,¹⁵⁵ β naphthylamine,¹⁵⁶ aniline,¹⁵⁷ ¹⁵⁸ ¹⁵⁹ *N* methyl *p* amino phenol¹⁶⁰ *p* methyl amino phenol sulfate¹⁶⁰ or others. A yellowish green color develops which is measured in one of the usual apparatus and can be extracted with amyl alcohol.¹⁵⁴ The color can be stabilized by a phosphate buffer of pH 6.1.¹⁶¹

¹⁴⁸ C E Bills F G McDonald and T D Spies *Southern Med J* 32 793 (1939)

¹⁴⁹ T D Spies A A Walker and A W Woods *J Am Med Assoc* 113 1481 (1933)

¹⁵¹ F C Schmelkes *Science* 90 113 (1939)

¹⁵³ H A Wassman O Mickelsen J M McKibbin and C A Elvehjem *J Nutrition* 19 483 (1940)

¹⁵⁴ W König *J prakt Chem* 69 105 (1904) 70 19 (1904)

¹⁵⁵ M Swaminathan *Nature* 141 830 (1938) H v Euler F Schlenk H Heiwinkel and B Högberg *Z physiol Chem* 256 208 (1938) G E Shaw and C A Macdonald *Quart J Pharm Pharmacol* 11 380 (1938) E Bandier and J Hald *Biochem J* 33 264 (1939)

¹⁵⁶ L J Harris and W D Raymond *J Soc Chem Ind* 58 652 (1939)

¹⁵⁷ H v Euler F Schlenk H Heiwinkel and B Högberg *Z physiol Chem* 256 208 (1938)

¹⁵⁸ H Kringstad and T Naess *Naturwissenschaften* 43 709 (1938) *Z physiol Chem* 260 108 (1939)

¹⁵⁹ M Swaminathan *Nature* 141 830 (1938) *Indian J Med Research* 26 427 (1938)

¹⁶⁰ D Melnick and H Field *J Biol Chem* 134 1 (1940) 135 53 (1940)

¹⁶¹ E Bandier and J Hald *Biochem J* 33 264 (1939) E Bandier *Ibid* 33 1130 (1939)

¹⁶² H Kringstad and T Naess *Naturwissenschaften* 26 709 (1938) *Z physiol Chem* 260 108 (1939)

It has been stated that the color developed with *p* amino acetophenone is more suited for quantitative determinations of minute amounts of nicotinic acid than the color from any of the other investigated amines. About 1 mg of nicotinic acid in a 1 g sample can be recognized by this procedure.¹⁶¹

The cyanogen bromide method is not specific for nicotinic acid. A number of other pyridine derivatives and especially derivatives of nicotinic acid such as trigonelline, nicotinuric acid and nicotine give similar color tests.¹⁶²

2 The 2,4-Dinitro-chlorobenzene Method¹⁶⁴ The material to be analyzed according to this method should contain only the free nicotinic acid or its amide. In most cases therefore, at least a partial hydrolysis must be carried out. The dry material is then fused with 2,4 dinitro chlorobenzene and the reaction product is dissolved in alcohol and potassium hydroxide is added. The color developed is measured colorimetrically.^{165, 166}

Neither of these two methods is specific for nicotinic acid as the same colors are given by many pyridine derivatives. During systematic studies of the nicotinic acid content of various plant and animal materials it has been observed that the materials, especially cereals, give color reactions with these two methods in amounts which cannot be explained on the basis of the amount of the nicotinic acid actually present.¹⁶⁷ Further inaccuracies arise from procedures in which nicotinic acid is extracted from other undesirable compounds and from attempts to prepare colorless extracts as is necessary for the determination of nicotinic acid in urine and in blood (see p. 247).

For the determination of nicotinic acid in tablets and in ampules a tentative A O A C method (Association of Official Agricultural Chemists) has been worked out¹⁶⁸ which consists in subliming preferentially the nicotinic acid out of the sample and by weighing or titrating¹⁶⁹ the sublimate.

¹⁶¹ I. Kodicek *Biochem J* 34 712-714 (1940)

¹⁶² D. Melnick, W. D. Robinson and H. F. Id. *J Biol Chem* 136 131 (1940)

¹⁶³ E. Vongerichte *Ber* 32 2571 (1899)

¹⁶⁴ P. Karrer and H. Keller *Helv Chim Acta* 21 463-1170 (1938) S. P. Vilter, T. D. Spies and A. P. Mathews *J Biol Chem* 125 85 (1938) *J Am Chem Soc* 60 731 (1938)

¹⁶⁵ H. v. Euler, F. Schlenk, L. Melzer and B. Höglberg *Z physiol Chem* 258 212 (1939) P. Karrer and H. Keller *Helv Chim Acta* 22 129 (1939)

¹⁶⁶ I. Kodicek *Biochem J* 34 712-721 (1940)

¹⁶⁷ *Methods of Analysis*: Assoc. Official Agricultural Chemists, 1940, p. 610 *J Assoc Official Agr Chem* 23 38 (1940)

¹⁶⁸ P. S. Jorgensen *J Assoc Official Agr Chem* 23 765 (1940)

Nicotinic acid is separated from its amide by ether extraction of a water solution. Only the amide is extracted by ether.

(b) Biochemical Methods

Lactobacillus Test In this test the amount of lactic acid produced by the *Lactobacillus arabinosus* is measured. When adequately supplied with all necessary growth factors, the quantity of lactic acid produced is directly proportional to the amount of nicotinic acid present.¹⁷⁰ This method has several advantages over the chemical methods of nicotinic acid assay. Turbidity and color of the sample to be tested do not interfere with the determination. Nicotinic acid and its amide have equal activities. The sensitivity of this test is greater than that of the chemical tests. One γ or less can be determined accurately.

(c) Biological Methods

None of the proposed biological methods listed below has been used extensively enough to claim quantitative determinations under all conditions. In earlier qualitative experiments dogs proved very valuable as test animals and it seems that some of the microorganisms will serve in the future for quantitative determinations. The following methods have been suggested:

- 1 Dog test^{171 172 173 174}
- 2 Chick test¹⁷²
- 3 Moth test (*Galleria melonella*)¹⁷⁵
- 4 Microorganism growth tests on *Staphylococcus aureus*^{176 177 178}
*Shigella paradysenteriae*¹⁷⁹ and *Bacterium proteus*¹⁸⁰

¹⁷⁰ E. E. Snell and L. D. Wright *Proc. Am. Soc. Biol. Chem.* 1941 CXLX

¹⁷¹ C. A. Elvehjem, R. J. Madden, F. M. Strong and D. W. Woolley *J. Am. Chem. Soc.* 59 1787 (1937) *J. Biol. Chem.* 123 137 (1938)

¹⁷² C. J. Koehn and C. A. Elvehjem *J. Biol. Chem.* 118 693 (1937)

¹⁷³ J. Goldberger, G. A. Wheeler, R. D. Lillie and L. M. Rogers *U. S. Pub. Health Service Pub. Health Repts.* 43 1385 (1928)

¹⁷⁴ W. H. Sebrell *Ibid.* 49 754 (1934) W. H. Sebrell, G. A. Wheeler and D. J. Hunt *Ibid.* 50 1333 (1935)

¹⁷⁵ D. Rubinstein and L. Shekun *Nature* 143 1064 (1939)

¹⁷⁶ M. Landy *Ibid.* 142 618 (1938)

¹⁷⁷ B. C. J. G. Knight and H. Mellman *Biochem. J.* 32 1241 (1938)

¹⁷⁸ B. C. J. G. Knight *Ibid.* 31 731 (1937)

¹⁷⁹ H. F. F. and N. H. Toising and W. H. Sebrell *U. S. Pub. Health Service Pub. Health Repts.* 53 1876 (1938)

¹⁸⁰ A. Lwoff and A. Quirido *Compt. rend. soc. biol.* 129 1032 (1938)

15 Standard of Nicotinic Acid and Nicotinamide

Up to the present time no national or international standard has been set up for nicotinic acid or nicotinamide. There is actually little need for such a standard since nicotinic acid and its amide are well known crystallized compounds which are used on the weight basis.

16 Physiology of Plants and Microorganisms

Nicotinic acid amide is a normal cell constituent and growth factor for plants and microorganisms. It is generally assumed that the coenzyme function in reversible oxidation-reduction systems represents the main activity of nicotinic acid, but some evidences have been brought forward which indicate that nicotinic acid or its amide may have an additional function outside the coenzyme linkages (see page 245).

The actual need of plants for nicotinic acid has been demonstrated on isolated pea embryos and isolated pea radish and cosmos roots.^{181, 182} Among the microorganisms are certain species which synthesize their own nicotinic acid requirements while others depend upon an external supply of this essential nutrient. Among the organisms which need nicotinic acid as a regular food constituent are certain bacteria such as *B. diphtheriae*,¹⁸³ *B. dysenteriae*,¹⁸⁴ *B. proteus*,¹⁸⁵ *Staphylococcus aureus*,^{186, 187, 188} lactic acid bacteria,¹⁸⁹ protozoa such as Protista¹⁹⁰ and certain fungi.¹⁸⁸ Still more parasitic organisms need an external supply of the cohydrogenases for proper growth which is not induced by the administration of the components of the coenzyme. The *B. influenzae* belongs to this group.¹⁹⁰

17 Animal Physiology

General Physiology, Metabolism and Mechanism of the Vitamin Action

The normal dietary intake of nicotinic acid consists mostly of the coenzymes which are present in food of plant and animal origin. It is assumed that the enzyme complex is split in the intestinal tract but it is uncertain if the coenzymes can be absorbed as such or if they are liq

J. Boune, *Pl. i. Phys.* 13, 86 (1938). F. T. Adlitt and J. Boune, *Science* 88, 57 (1938).

J. Boune, *Am. Chem. Soc. Div. Agr. Food Chem. Meet. g. Sept. 1939*, Abstr. 13, 14.

J. H. Mueller, *J. Bact.* 34, 421 (1937). *J. Biol. Chem.* 120, 219 (1937).

¹ S. A. K. v. A. Doerflinger and P. Sauder, *Proc. Soc. Exptl. Biol. Med.* 38, 311 (1938). 5, 90 (1932).

² F. F. Iles, *Brit. J. Food Hyg.* 10, 239 (1938).

³ B. C. J. C. Knight, *Biochem. J.* 31, 741 (1937).

⁴ M. Laury, *Nat.* 142, 618 (1918).

⁵ B. C. J. C. Knight and H. M. Liaw, *Biochem. J.* 32, 1211 (1938).

⁶ F. F. Smith, F. M. Strong and W. H. Peterson, *J. Am. Chem. Soc.* 60, 282 (1938).

⁷ R. W. Vilter, F. V. Vilter and T. D. Spence, *J. Am. Med. Assoc.* 112, 420 (1939).

hydrolyzed prior to the absorption. Nicotinic acid and its amide are absorbed unchanged. Nicotinic acid is amidated in the organism after absorption by the blood stream. The nicotinic acid and its amide are transported in the blood serum, which maintains a certain level of this essential compound, but contains no coenzymes. The blood corpuscles, on the other hand, contain relatively high concentrations of the coenzymes but no free nicotinamide.

There are no special storage organs for nicotinic acid or any of its derivatives. In the form of the coenzymes, nicotinic acid is present in practically all cells. While nicotinic acid deficiency in the dog and pig results in a lowered coenzyme content of the liver and muscles^{191 192 193} no substantial effect has been noted upon the coenzyme content of the brain, kidney cortex and blood. In human beings nicotinic acid deficiency causes a marked decrease in the coenzyme content of striated muscles but has only a slight effect upon the coenzyme content of the erythrocytes.¹⁹⁴

Nicotinic acid is, like all the other vitamins, secreted in milk and is found in eggs.

Nicotinic acid and its metabolic end products are excreted through the urine. The normal human organism excretes a certain amount in the free form. Coenzymes are not excreted.^{195 196} Ingested nicotinic acid is excreted in considerable amounts in the form of nicotinuric acid and trigonelline. There is, furthermore, apparently a combined form of nicotinic acid in the urine¹⁹⁷ which may be different from nicotinuric acid. The total output depends upon the intake. Normally about 4–5 mg are excreted daily.¹⁹⁸ Lowered values have been observed in pellagrins and during times of anorexia. Guinea pigs deprived of nicotinic acid show a progressive decrease of nicotinic acid excretion which reaches a zero value at the time when clinical deficiency symptoms develop. Dogs excrete only trigonelline and nicotinuric acid but no nicotinic acid or its amide.¹⁹⁹ Rabbits, on the other hand, cannot synthesize trigonelline from nicotinic acid.²⁰⁰

Nicotinic acid appears to function mainly as a part of enzyme systems which have been discussed previously and which take part in the protein

¹⁹¹ A. E. Axelrod, R. J. Madden and C. A. Elvehjem, *J. Biol. Chem.* **131**, 85 (1939).

¹⁹² H. I. Kohn, J. R. Klein and W. J. Dann, *Biochem. J.* **33**, 1432 (1939).

¹⁹³ M. Pittman and H. F. Frazer, *U. S. Pub. Health Service Pub. Health Repts.* **55**, 915 (1940).

¹⁹⁴ A. E. Axelrod, T. D. Spies and C. A. Elvehjem, *J. Biol. Chem.* **138**, 687 (1941).

¹⁹⁵ R. W. Vilter, S. P. Vilter and T. D. Spies, *J. Am. Med. Assoc.* **112**, 4.0 (1939).

¹⁹⁶ H. v. Euler, F. Schlenk, H. Heiwinkel and B. Högborg, *Z. physiol. Chem.* **256**, 208 (1938).

¹⁹⁷ E. Bandier, *Biochem. J.* **33**, 1787 (1939).

¹⁹⁸ L. J. Harris and W. D. Raymond, *J. Soc. Chem. Ind.* **58**, 652 (1939).

¹⁹⁹ D. Ackermann, *Z. Biol.* **59**, 17 (1912).

²⁰⁰ W. A. Perlzweig, H. P. Sarett and J. W. Huff, *Proc. Am. Soc. Biol. Chem.* **1941**, C.

and the carbohydrate metabolism by transporting hydrogen. At birth the tissues of mammals contain only small amounts of the nicotinamide coenzymes I and II (about 100-150 γ per gram of liver or kidney in rats), but the amount rises rapidly and reaches the normal range of adults (about 250 γ in rats) in about seven days.⁶¹

There is the possibility that nicotinic acid may act in other functions than through the coenzymes I and II. Thus nicotinic acid deficiency causes water retention and may therefore be linked with the water metabolism.⁶² This effect could, however, be understood as a side reaction of the disturbance of the carbohydrate metabolism. Nicotinic acid also influences the metabolism of the heavy metals. Also in this case as in the influence upon the water metabolism, no decision can be made on the basis of the experimental facts as to whether this is a primary action of nicotinic acid, its amide or the coenzymes or if these disturbances are secondary reactions. A pigmentation in the skin and the mucous membranes of the mouth is often observed in a human being suffering from a nicotinic acid deficiency. The pigment is not melanin but an iron pigment.⁶³ As the result of a nicotinic acid deficiency a type of anemia occurs which responds favorably to iron therapy. The iron containing porphyrins undergo some kind of decomposition and as the result of this an increased amount of porphyrin compounds is secreted through the urine.⁶⁴ The copper and iron containing enzyme systems such as cytochrome, the polyphenol oxidases, the peroxidase and catalase appear to be affected. Thus for example a disturbance of the tryptophane catabolism has been observed in pellagrins which resulted in the excretion of indoxyl ethyl amin.⁶⁵ The disturbances of the sympathetic nervous system could perhaps be explained as a disturbed relation of the polyphenol oxidases to adrenalin.

Nicotinic acid decreases the peristaltic action of the stomach and small intestine while inositol (see page 279) increases this action. The other members of the B complex have no apparent action of this type. It has therefore been suggested that the balance or ratio of nicotinic acid to inositol is the nutritional factor which determines hypo- or hypergastrointestinal motility.⁶⁶

There are observations which indicate functions of nicotinic acid which cannot be explained on the basis that nicotinic acid acts only through the

⁶¹ F. B. Ruhe and A. v. Felsoranyi, *Science* 91, 76 (1940).

⁶² C. Funk and T. C. Funk, *Z. Vitaminforsch.* 8, 330 (1938).

⁶³ H. Herzog, *g. Beitr. path. Anal.* 96, 37 (1935).

⁶⁴ T. D. Spiess, W. B. Bean and R. F. Stone, *J. Am. Med. Assoc.* 111, 584 (1938).

⁶⁵ M. C. Sullivan, *J. Biol. Chem.* 50, 39 (1922).

⁶⁶ G. J. Martin, M. R. Thompson and J. de Carvalhal Forero, *Am. J. Digest. Dis.* 8, 290 (1941).

coenzymes Blacktongue in dogs can be cured easily by nicotinic acid, but no significant effect could be noted when cozymase was injected intravenously in an amount which on the basis of its nicotinic acid content would have been expected to exert beneficial effects²⁰⁶ Growth and respiration of dysentery bacilli are much more favorably influenced by nicotinic acid or its amide than by the coenzymes, and the efficacy of the action of the coenzymes can be increased markedly by hydrolysis under conditions which free the nicotinamide²⁰⁷⁻⁰⁸

18 Avitaminosis

There are no clinical symptoms known for slight nicotinic acid deficiency in man Definite diagnostic evidences develop late²⁰⁹ In severe cases the blood level of nicotinic acid is decreased This has frequently been observed²¹⁰ in pregnant women on diets low in nicotinic acid, since during pregnancy increased amounts of this vitamin are needed Another relatively early symptom of nicotinic acid deficiency is the excretion of porphyrins in the urine.²¹¹

The typical symptoms of nicotinic acid deficiency in man are commonly summarized in the term pellagra The typical pellagrin shows characteristic lesions of the mucous membranes, for example in the mouth (glossitis), and of the skin over the nose forehead, dorsum hands wrists elbows knees and feet This type of dermatitis involves especially those parts of the body which are exposed to sunlight or to friction Disturbances of the gastrointestinal tract are observed in many cases In later stages of the disease, mental disorders and lesions of the central nervous system occur, which are characterized by clouding of consciousness cogwheel rigidities and uncontrollable grasping and sucking reflexes¹ A certain form of anemia has been observed in severe cases Diagnosis of pellagra is sometimes very difficult since the symptoms described do not necessarily occur all at once

Typical pellagra is, however, not a disease caused solely by a nicotinic acid deficiency Usually pellagra is the result of a multiple vitamin deficiency and can be cured only by the administration of several or all

²⁰⁶ F S Daft H F Frazer W H Sebrell and M Pittman *Science* 88 128 (1938)

²⁰⁷ A Dorfman S A Koser H R Reames K F Swingle and F Saunders *Proc Soc Exptl Biol Med* 43 163 (1940) F Saunders A Dorfman and S A Koser *J Biol Chem* 138 69 (1941) 1

C Norris and A T Ringrose *Science* 71 643 (1930)

²⁰⁸ M A Blankenhorn and T D Spies *J Am Med Assoc* 108 583 (1931)

²¹⁰ A I woff A Querido L Dignonnet and Carmer *Compt rend soc biol* 131 900 (1933)

²¹¹ T D Spies W B Bean and R F Stone *J Am Med Assoc* 111 584 (1938)

²¹² N Jolliffe K M Bowman I A Rosenblum and H D Fern *Ibid* 114 307 (1940)

members of the vitamin B complex. Besides nicotinic acid, riboflavin and thiamin therapy are especially necessary.^{213 214}

Nicotinic acid treatment has been observed to give beneficial results to pneumonia patients who under sulfanilamide therapy have developed a black dotted heavy furring of the tongue which resembles the blacktongue of dogs.²¹⁵

The lesions in the mouth caused by nicotinic acid deficiency sometimes are very similar in appearance to the lesions of Vincent's infection but are genetically different. It seems that Vincent's infection frequently sets in when the mucous membranes are weakened due to nicotinic acid deficiency and in such cases a treatment with nicotinic acid is, of course, beneficial.²¹⁶ Encouraging results have been obtained by administration of nicotinic acid for the prevention and treatment of irradiation (x ray) sickness.²¹⁷ Furthermore it has been suggested²¹⁸ that nicotinic acid may be of value in the treatment of eighth nerve (high tone) deafness.

In dogs the typical syndrome of nicotinic acid deficiency is the occurrence of blacktongue. In adrenalectomized rats a special form of dermatitis has been noted which responds favorably to treatment with nicotinic acid.²¹⁹

(a) Clinical Test Methods

The methods which have been suggested for the determination of a state of nicotinic acid deficiency comprise the determination of the amount excreted in urine or present in blood. In addition, the occurrence of porphyrins in the urine can be used as an additional test method.

Urine Tests The determination of the excretion of nicotinic acid is difficult since urine contains this compound not only in the free form but also in the form of various derivatives mainly as nicotinamide, nicotinic acid and trigonelline. In order to obtain uniform results, the urine must be hydrolyzed, preferably with alkali.²²⁰ The color present in urine invariably interferes with colorimetric determinations for the nicotinic acid content. Preferential adsorption of the colored materials on

²¹³ G. Margolis, L. H. Margolis and S. G. Smith, *J. Nutrition* 16: 541 (1938); 17: 63 (1939). O. M. Helmer and P. J. Fouts, *Ibid.* 16: 271 (1938). W. H. Sebrell, *J. Am. Med. Assoc.* 110: 1665 (1938).
²¹⁴ W. Viter, S. P. Viter and T. D. Spe, *Ibid.* 112: 420 (1939).

²¹⁵ R. W. Viter, S. P. Viter and T. D. Spe, *J. Am. Med. Assoc.* 112: 40 (1939).

²¹⁶ F. M. Josephson and O. Klewan, *Nature* 143: 770 (1939).

²¹⁷ W. Sophi, *Am. J. Digestive Diseases Nutrition* 7: 298 (1940). J. D. King, *Lancet* 2: 32 (1940).

²¹⁸ J. W. Crumham, *J. Am. Med. Assoc.* 113: 664 (1939).

²¹⁹ G. Sell, *Ann. Otol. Rhin. Laryng.* 48: 39 (1939).

²²⁰ L. Laszt, *Z. Vitaminforsch.* 11: 76 (1941).

²²¹ F. Bendier, *Biochem. J.* 33: 1787 (1939).

charcoal or on zinc hydroxide²²¹ has been suggested. None of the color reactions can be considered reliable since urine contains substances which interfere with the determination. As the result of these difficulties, only approximate values can be expected from the determination of nicotinic acid in urine. For the actual test, the color reaction with 2,4-dinitrochlorobenzene has been modified for urine analysis.²²² The hydrolyzed and decolorized urine is evaporated and the dry residue is mixed with 2,4 dinitro chlorobenzene in alcohol, and after standing for several hours the alcohol is distilled off and the residue is heated to 105° C for 10 minutes. After cooling, a solution of KOH in alcohol is added. A purple color develops which can be determined by the usual methods. The cyanogen bromide method has frequently been used for the determination of nicotinic acid in urine and a number of slight modifications of the basic technic have been recommended.²²³⁻²²⁶ Both of these methods are unreliable because other compounds, for example, nicotine and trigonelline, give similar color reactions.²²⁷

A somewhat better method for the determination of a deficiency is to perform an *excretion test* on the patient.²²⁸ Excess doses of nicotinic acid or its amide are given and the amount excreted is determined before and after the ingestion. The total amount of compounds which behave like nicotinic acid in the color tests varies considerably in normal persons²⁹ and is dependent on many factors. Thus, smokers usually excrete increased amounts of chromogenic material. Upon oral administration of, for example 500 mg of nicotinic acid to normal individuals, the urinary output rises to its maximum within one hour and drops to normal in about four hours. In a series of experimental tests it was found that from the ingested 500 mg about 110 mg are excreted by normal persons. Of this total amount about 51% is present as trigonelline, 36% as nicotinuric acid and 13% as free nicotinic acid or nicotinamide. Coenzymes are normally not excreted.

A state of nicotinic acid deficiency can also be suspected from the *excretion of porphyrins through the urine*, but it should be remembered that

²²¹ T. E. Friedemann and C. J. Barborka *J. Biol. Chem.* **138**, 787 (1941).

²²² P. Karrer and H. Keller *Helv. Chim. Acta* **21**, 463, 1170 (1938). S. P. Vilter, T. D. Spies and A. P. Mathews *J. Biol. Chem.* **125**, 85 (1938); *J. Am. Chem. Soc.* **60**, 731 (1938).

²²³ E. Bandier *Biochem. J.* **33**, 1787 (1939).

²²⁴ L. J. Harris and W. D. Raymond *Ibid.* **33**, 2037 (1939).

²²⁵ D. Meinick and H. Field *J. Biol. Chem.* **134**, 1 (1940).

²²⁶ L. A. Rosenblum and N. Jolliffe *Ibid.* **134**, 137 (1940).

²²⁷ D. Meinick, W. D. Robinson and H. Field *Ibid.* **136**, 131 (1940).

²²⁸ D. Meinick, W. D. Robinson and H. Field *Ibid.* **136**, 145 (1940).

²²⁹ D. Meinick, W. D. Robinson and H. Field *Ibid.* **136**, 157 (1940).

porphyruria may have other causes than a nicotinic acid deficiency. In the actual test, the urine is acidified with acetic acid and extracted with ether. The ether solution is washed with water and then with 25% hydrochloric acid. The latter causes the appearance of a color, which indicates the presence of porphyrins.²³⁰

Blood Tests Blood contains both nicotinic acid (or its amide) and the codehydrogenases. The latter are almost completely confined to the blood corpuscles^{231 22 33} while the serum contains only the free nicotinic acid (or its amide). Of the total amount about 90% is in the erythrocytes and this amount decreases only very slightly at times of nicotinic acid deficiency. The small amount of free nicotinic acid present is a function of the intake. Upon oral administration of nicotinic acid to fasting persons the blood level increases promptly but returns rapidly to values only slightly higher than the basal level.²³² The total nicotinic acid content of the blood of normal persons ranges between 0.52 and 0.83 mg %²³³

The actual determination of the nicotinic acid (or its amide) can be carried out by means of the *cyanogen bromine method*,^{234 235 236 237 238} by the *Bacillus proteus growth method*²³⁹ and the codehydrogenase I can be determined by the *fermentation method*.²⁴⁰

19 Hypervitaminosis

The intake of about 1000 times the amount of nicotinic acid which is normally consumed in food may be considered as relatively non toxic. Higher doses however exhibit typical toxicological symptoms. Dogs kept at a daily intake of 2 g. of nicotinic acid died within twenty days. In human beings the oral administration of large quantities of nicotinic acid is followed by flushing, burning, itching and increased sensations of local heat in the skin. Nicotinic acid amide does not produce these symptoms⁴¹ and is therefore recommended for clinical use.

²²⁸ T. D. Spies, R. W. Vilter and W. F. Ashe, *J. Am. Med. Assoc.* 113, 931 (1935).

²³¹ A. Doisman, M. K. Horwitz, S. A. Koser and F. S. Under, *Proc. Am. Soc. Biol. Chem.* 128, XX (1939).

²³² H. I. Fohn and J. R. Klein, *J. Biol. Chem.* 130, 1 (1939).

²³³ D. Melnick and W. D. Robinson and H. Feld, *Ibid.* 136, 157 (1940).

²³⁴ D. Melnick and H. Feld, *Ibid.* 134, 1 (1940); 135, 53 (1940).

M. Swaminathan, *Indian J. Med. Research* 26, 427 (1938).

²³⁶ P. B. Parson, *J. Biol. Chem.* 129, 491 (1939).

²³⁷ K. R. Sert, *Klin. Wochschr.* 18, 934 (1939).

²³⁸ T. D. Spies, A. A. Walker and A. W. Woods, *J. Am. Med. Assoc.* 113, 1481 (1939).

²³⁹ A. Querido, M. Albeaux, Fe. net and A. Lwoff, *Compte rend. soc. biol.* 131, 18 (1939). A. Querido, A. Lwoff and C. Latast, *Ibid.* 130, 1580 (1939).

²⁴⁰ A. E. Axelrod and C. A. Elv. hjem, *J. Biol. Chem.* 131, 77 (1939).

²⁴¹ T. D. Spies, D. P. Hightower and L. H. Hubbard, *J. Am. Med. Assoc.* 115, 29 (1940).

20 Nicotinic Acid Requirements

It is established that all living tissues need nicotinic acid. Some organisms need an external supply of this vitamin while others are able to synthesize it.

The daily requirements of man are of the order of 12–23 mg (Details see page 613). Therapeutically, nicotinic acid has been given orally in daily doses ranging from 50 to 500 mg. Monkeys apparently need about 5 mg daily.⁴ The necessity of a regular intake of nicotinic acid has also been proved for dogs,¹³ pigs⁴¹ and the moth (*Galleria melonella*).²⁴⁵

Certain species of microorganisms are able to synthesize nicotinic acid such as, for example, *Chilomonas paramecium*,²⁴⁶ while other species, such as *Bacterium pneumococcus*,²⁴⁷ need an external supply of nicotinic acid for proper growth.^{248, 249} Some other unicellular organisms, for example the *Bacillus influenzae*,²⁵⁰ do not grow upon administration of nicotinic acid or its amide, but need codehydrogenase I or II. Some of the microorganisms which are able to synthesize nicotinic acid have been shown to live in the intestinal tract of higher animals, such as, for example, in sheep⁵¹ and in cattle.⁵²

The nicotinic acid requirement of the rat has been the subject of extensive discussion. While it is certain that the rat needs this vitamin,^{53, 254, 255} the question as to whether or not the rat must have nicotinic acid in its daily food intake is not settled. It has been suggested that the rat is able to synthesize its own supply,²⁵⁶ but it seems more plausible to assume that if rats do not need to have nicotinic acid in their food, they have microorganisms living in their intestinal tract which synthesize this vitamin and from which the rats obtain the necessary amount.

¹³ L. J. Harris *Biochem J.* **32**, 1479 (1938).

⁴¹ C. A. Elvehjem, R. J. Madden, F. M. Strong and D. W. Woolley *J. Am. Chem. Soc.* **59**, 176 (1937); *J. Biol. Chem.* **123**, 137 (1938).

⁴ H. Chuck, T. F. Macrae, A. J. P. Martin and C. J. Martin *Biochem J.* **32**, 10 (1938).

²⁴⁶ D. Rubinstein and L. Shekua *Nature* **143**, 1064 (1939).

²⁴⁷ J. O. Hutchens, B. J. Jandorf and A. B. Hastings *J. Biol. Chem.* **138**, 321 (1941).

²⁴⁸ L. Rane and Y. Subbarow *Ibid.* **134**, 455 (1940).

²⁴⁹ B. C. J. G. Knight *Biochem J.* **31**, 731 (1937).

²⁵⁰ J. H. Mueller *J. Bact.* **34**, 429 (1937); *J. Biol. Chem.* **120**, 219 (1937).

⁵¹ A. Lwoff and M. Lwoff *Proc. Roy. Soc. (London)* **B122**, 352, 360 (1937); *Compt. rend.* **203**, 520 (1936).

⁵² A. H. Winegar, P. B. Parson and H. Schmidt *Science* **91**, 508 (1940).

⁵³ M. I. Wegner, A. N. Booth, C. A. Elvehjem and E. B. Hart *Proc. Soc. Exptl. Biol. Med.* **45**, 769 (1940).

²⁵⁴ D. V. Frost and C. A. Elvehjem *J. Biol. Chem.* **121**, 255 (1937); I. György *Proc. Soc. Exptl. Biol. Med.* **37**, 732 (1938); W. R. Wyatt *Iowa State Coll. J. Sci.* **14**, 103 (1939).

²⁵⁵ H. v. Fuler, F. Schlenk, I. Melzer and B. Högberg *Z. physiol. Chem.* **258**, 112 (1949).

²⁵⁶ I. Laszt *Z. Vitaminforsch.* **11**, 76 (1941).

²⁵⁷ W. J. Dann and H. I. Kohn *J. Biol. Chem.* **136**, 435 (1940).

**PANTOTHENIC
ACID**

PANTOTHENIC ACID

1 Nomenclature and Survey

Names

- Pantothenic acid¹ (the name is derived from the Greek meaning from everywhere)
- Pantothen Abbreviated term
- Antidermatosis vitamin²
- Chick antidermatitis factor³
- Vitamin B⁴
- Filtrate factor (liver filtrate factor⁵ yeast filtrate factor⁶)
- Factor 2 (from liver)⁷
- Chick A P factor (Chick Anti pellagra factor)⁸ The term pellagra is properly used only in describing human diseases (in compliance with the Committee on Vitamin Nomenclature)
- Vitamin B₂ (G)⁹ (abandoned historical name)

Probably also identical with

- Vitamin B₂ Vitamin B₂ is a term given in 1928 to a heat labile factor necessary for weight maintenance of pigeons^{10 11} Recent findings point to the identity of this vitamin with pantothenic acid¹²

¹ R J Williams C M Lyman C H Goodyen T H Truested and D Holaday *J Am Chem Soc* 55 2312 (1933)

² J C Bauernfeind A B Schumacher A Z Hodson L C Norris and G F Heuser *Proc Soc Exptl Biol Med* 39 108 (1938)

³ O Mickelsen H A Waisman and C A Elvehjem *J Biol Chem* 124 313 (1938) D W Woolley H A Waisman O Mickelsen and C A Elvehjem *Ibid* 125 715 (1938)

⁴ A Bakke V Aschehoug and C Zbinden *Compt rend* 191 1157 (1930) A F Morgan B B Cook and H C Davison *J Analysis* 15 7 (1938) A F Morgan and H D Simms *Ibid* 19 233 (1940) G Lunde and H Kringstad *Arch Norske Vid Akad Oslo I Math Naturw Klasse No 1* 1 (1938) *Z physiol Chem* 257 01 (1939) 261 110 (1939) *Naturwissenschaften* 27 755 (1939) *Angew Chem* 52 521 (1939) G Lunde H Kringstad and E Jansen *Naturwissenschaften* 29 62 (1941)

⁵ T H Jukes and S Lepkovsky *J Biol Chem* 111 119 (1935) 114 109 117 (1936)

⁶ C E Edgar and T F M erce *Biochem J* 31 886 893 (1937)

⁷ S Lepkovsky T H Jukes and M E Krause *J Biol Chem* 115 557 (1936)

⁸ C J Koehn and C A Elvehjem *Ibid* 118 693 (1937)

⁹ C A Elvehjem and C J Koehn *Nature* 134 1007 (1934) *J Biol Chem* 108 709 (1935)
The authors suggested that the chick pellagra preventative factor be called vitamin B₂ or G in differentiation from riboflavin. Since however this vitamin is now called vitamin B₂ or G the utilization of these letters to designate pantothenic acid has been abandoned. For a discussion of this nomenclature see also ref 5 and C J Koehn and C A Elvehjem *J Nutrition* 11 67 (1936)

¹⁰ R R Williams and R E Waterman *J Biol Chem* 78 311 (1928)

¹¹ C. W Carter and J R O'Brien *Biochem J* 30 43 (1936)

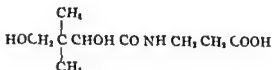
¹² C. W Carter and J R O'Brien *Ibid* 33 1810 (1939)

The spectacled eye condition prevents factor for rats. The spectacled eye condition has been described¹³ as an erosion occurring around the eyes of rats and imparting to the animals a spectacled appearance. The lids became denuded and scaly. In more severe cases the eyes are closed by a sticky exudate. Since concentrates of pantothenic acid brought about a rapid cure of the spectacled eye condition it is supposed¹⁴ that the two factors may be identical. (See however pages 280 and 477.)

Empirical formula



Structural formula



Chemical name

(+)- α - γ Dihydroxy β - β dimethyl butyryl β' alamide

Efficacy

1 g of pantothenic acid = 70 000-75 000 Chick Units

2 Chronology

- 1930 NORRIS and RINGROSE¹⁵ described a specific dermatosis of chicks
 1933 WILLIAMS and co workers¹⁶ found that a naturally occurring compound of unknown chemical composition called pantothenic acid stimulates the growth of yeast
 1934-1935 ELVEHJEM and LOFFEN¹⁷ differentiated the chick antidermatitis factor from riboflavin and cured the chick syndrome with liver extract
 1936 LEPKOVSKY, JUKES and KRAUSE showed the need of rats for a factor distinct from thiamin, riboflavin and vitamin B₆¹⁸
 1937 FOUTS, LEPKOVSKY, HELMER and JUKES¹⁹ and independently DANN²⁰ stated that nicotinic acid does not cure or prevent chicken dermatitis

¹ J. Goldberger and R. D. Lillie *U. S. Pub. Health Service Pub. Health Repts.* 41: 1025 (1926)
 A. Bourquin and H. C. Sherman *J. Am. Chem. Soc.* 53: 3501 (1931) H. E. Robinson and R. C. Newton Abstracts Division of Biological Chemistry *Am. Chem. Soc. Kansas City* April 13-17 (1934) S. Lepkovsky, T. H. Jukes and M. E. Krause *J. Biol. Chem.* 115: 557 (1936)

² J. J. Oleson, H. R. Bird, C. A. Elvehjem and E. B. Hart *J. Biol. Chem.* 127: 23 (1939)

¹⁵ I. C. Norris and A. T. Ringrose *Science* 71: 643 (1930) A. T. Ringrose, I. C. Norris and G. F. Heuser *Poultry Sci.* 10: 166 (1931)

¹⁶ R. J. Williams, C. M. Lyman, G. H. Goodyear, T. H. Truesdail and D. Holaday *J. Am. Chem. Soc.* 55: 2912 (1933)

¹⁷ C. A. Elvehjem and C. J. Koehn *Nature* 134: 1007 (1933) *J. Biol. Chem.* 108: 703 (1933)

¹⁸ S. I. Lepkovsky, T. H. Jukes and M. E. Krause *J. Biol. Chem.* 115: 557 (1936)

¹⁹ F. J. Fouts, S. I. Lepkovsky, O. M. Helmer and T. H. Jukes *Proc. Soc. Exptl. Biol. Med.* 37: 403 (1937)

²⁰ W. J. Dann *Science* 86: 616 (1937)

- 1938 The chemical nature of pantothenic acid was worked out by R. J. Williams and co workers ²¹
- 1939 The identity of pantothenic acid and the chick antidermatitis factor was recognized by Jukes²² and by Woolley, Waismán and Elvehjem ²³
- 1940 Pantothenic acid was totally synthesized by Stiller, Harris, Finkelstein, Kereztzsy and Folkers²⁴ and independently by Reichstein and Grüssner ²⁵ and by Kuhn and Weidt ²⁶

3 Occurrence

Pantothenic acid occurs in all types of animal tissues and seems to be present universally in protoplasm. The liver and kidney are the richest known animal sources of pantothenic acid followed by heart, spleen, brain, pancreas, tongue and lung ²⁷ the muscular tissue (of beef, lamb, pork and veal) contains considerably less. Pantothenic acid is produced by various molds and microorganisms²⁸ and by green plants after they develop their photosynthetic apparatus. The storage organs in plants, for example rice bran, appear to be especially rich in pantothenic acid ²⁹. Molasses³⁰ contains considerably more than normal plant tissue, for example alfalfa ³⁰.

In animal tissue (and probably also in plant tissue) pantothenic acid occurs free only to a very small extent but is usually found chemically bound to protein material ³¹.

4 Isolation

The isolation of pantothenic acid consists for example when using liver as the starting material, of the following steps ³². First the liver is allowed to autolyze in water. The water suspension is then heated and filtered from coagulated, inactive material. In place of this step alcoholic liver extracts may also be used as starting material. By treatment with fuller's earth much inactive, especially basic material is removed from the water

¹ R. J. Williams, H. H. Weinstock, L. Rohrmann, J. H. Truesdale, H. K. Mitchell and C. E. Meyer *J. Am. Chem. Soc.* **61**, 454 (1939).

²¹ T. H. Jukes *Ibid.* **61**, 975 (1939).

²³ D. W. Woolley, H. A. Waismán and C. A. Elvehjem *Ibid.* **61**, 977 (1939).

²⁴ E. T. Stiller, S. A. Harris, J. Finkelstein, J. C. Kereztzsy and K. Folkers *Ibid.* **62**, 1785 (1940).

²⁵ T. Reichstein and A. Grüssner *Helv. Chim. Acta* **23**, 50 (1940).

²⁶ R. Kuhn and T. Weidt *Ber.* **71**, 971, 1134 (1938).

²⁷ H. A. Waismán, O. Mykleson and C. A. Elvehjem *J. Nutrition* **18**, 47 (1939).

²⁸ C. H. McBurney, W. B. Bollen and R. J. Williams *Proc. Natl. Acad. Sci. U. S. A.* **21**, 301 (1937).

²⁹ R. J. Williams and R. Moser *J. Am. Chem. Soc.* **56**, 163 (1934).

³⁰ T. H. Jukes *J. Biol. Chem.* **114**, 11 (1937).

³¹ R. Kuhn and C. Weidt *Ber.* **71**, 780 (1938).

³² R. J. Williams, J. H. Truesdale, H. H. Weinstock, L. Rohrmann, C. M. Lyman and C. H. McBurney *J. Am. Chem. Soc.* **60**, 2719 (1938).

solution Pantothenic acid is then adsorbed on Norite at a pH of approximately 3.6 and immediately afterwards eluted³³ with ammonium hydroxide solution. A second adsorption on Norite may be carried out³³ followed by elution with a mixture of pyridine and methanol.

After neutralization with oxalic acid, the brucine salt of pantothenic acid is prepared and is extracted with chloroform. The crude brucine salts are fractionated by various distributions between chloroform and water. These salts are converted into the corresponding calcium salts by treating in solution with an excess of lime water and freeing from brucine by filtration and repeated extraction with chloroform. Purification of the calcium pantothenate consists in the precipitation of the active principle with alcohol, removal of some impurities by precipitation with mercuric chloride, fractional precipitation of the calcium salt with isopropyl ether and fractional precipitation from pyridine solution with acetone.

By application of these methods, about 3 g of crude, approximately 40% pure material were obtained from 250 kg of liver.^{32, 34} A modification³⁵ of this procedure is to precipitate impurities from the concentrated eluates of the charcoal adsorption with barium hydroxide until a pH of about 8 is reached and to dissolve the precipitated barium salt of pantothenic acid in absolute alcohol. After filtration the barium salt of the vitamin is obtained by concentrating the filtrate and by adding acetone, thus precipitating the acetone insoluble barium salts. The free acids are obtained from the barium salts by means of sulfuric acid and can be extracted with ether or amyl alcohol. Thus a concentrate containing approximately 20-25% pantothenic acid is obtained.

A somewhat different method consists³⁵ in freeing a liver (for example, tuna fish liver) extract from the fat soluble material and precipitating some impurities with mercuric acetate. The active material is then adsorbed on charcoal and eluted with a pyridine-methanol-water mixture. Impurities are removed from the eluate by precipitation with phosphotungstic acid and uracil-*d* riboside (uridine) is removed from the concentrated filtrate by crystallization from methanol. The pantothenic acid is then precipitated from the methanol solution with barium hydroxide. Further purification can be achieved by repeated precipitation (of impurities) with phosphotungstic acid. The active compound is then adsorbed on aluminum oxide, which has been activated with hydrochloric acid.

³² H. K. Mitchell, H. H. Weinstock, F. F. Snell, S. R. Stanbery and R. J. Williams, *J. Am. Chem. Soc.* **62**, 1776 (1940).

³⁴ V. Subbarow and G. H. Hitchings, *Ibid.* **61**, 1615 (1939).

³⁵ R. Kuhn and T. Wieland, *Ber.* **73**, 962 (1940).

5 Properties

Pantothenic acid is predominantly of acid character but shows also some basic properties^{36 37} The vitamin is readily soluble in water ethyl acetate, dioxane, glacial acetic acid, etc., somewhat soluble in ether and amyl alcohol³³ and practically insoluble in benzene, chloroform etc³³ The compound is highly hydrophilic and can be adsorbed on charcoal³⁷ but not on fuller's earth^{37 39} The acetyl derivative can be distilled at approximately 10^{-5} mm Hg³⁵ The vitamin is sensitive toward acids, bases and heat

Pantothenic acid in pure form is a pale yellow viscous oil The vitamin is dextrorotatory showing $[\alpha]_D^{25} +37.5^\circ$ It forms a microcrystalline calcium salt, $[\alpha]_D^{25} +24.3^\circ$ ⁴⁰

6 Chemical Constitution

Pantothenic acid has a molecular weight of 219 (values obtained experimentally are approximately 200^{41 42}) and has the empirical formula $C_8H_{17}O_5N$ ⁴² The presence of a carboxyl group in the molecule of this vitamin was proved⁴² by esterification with both diazomethane and methanol and recovery of the free acid by careful saponification Thus two of the five oxygens of the suggested empirical formula are accounted for Two other oxygens are present in the form of free hydroxyl groups⁴³ The presence and number of hydroxyl groups are suggested by the hydrophilic nature of pantothenic acid and of its methyl ester The ester is even more soluble in water than in ether Pantothenic acid is esterified by various acids and acid halides and thereby loses the vitamin activity which can be recovered by careful saponification A determination of the active hydrogen atoms suggested the presence of two hydroxyl groups Further evidence for the existence of at least two hydroxyl groups is based upon the observation that pantothenic acid condenses reversibly with acetaldehyde,

³⁶ R. J. Williams and D. H. Saunders *Biochem J* 28 1887 (1934) O. W. Richard *J Biol Chem* 113 531 (1936)

³⁷ R. J. Williams, J. H. Truesdail, H. H. Wentstock, E. Rohrmann, C. M. Lyman and C. H. McBurney *J Am Chem Soc* 60 2719 (1938)

³⁸ D. W. Woolley, H. A. Waisman, O. Mickelson and C. A. Elvehjem *J Biol Chem* 125 715 (1938)

³⁹ S. Lepkovsky, T. H. Jukes and M. E. Krause *Ibid* 115 507 (1936)

⁴⁰ E. T. Stiller, S. A. Harris, J. Finkelstein, J. C. Keresztesy and K. Folkers *J Am Chem Soc* 62 1785 (1940)

⁴¹ R. J. Williams, C. M. Lyman, G. H. Goodyear, T. H. Truesdail and D. Holaday *Ibid* 53 912 (1933)

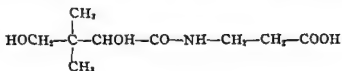
⁴² R. J. Williams, H. H. Wentstock, E. Rohrmann, J. H. Truesdail, H. K. Metcalf and C. E. Meyer *Ibid* 61 454 (1939)

⁴³ R. J. Williams and R. Moser *J Am Chem Soc* 56 169 (1934)

acetone and benzaldehyde. This type of reaction suggests the presence of an α, β , α, γ or α, δ glycol. The behavior of pantothenic acid in an electrical field⁴³ indicated the ionization constant to be approximately 3.9×10^{-4} . This corresponds in strength approximately to that of a β or γ hydroxycarboxylic acid. The absence of a hydroxyl group in α position to the carboxyl group is also suggested by the failure of pantothenic acid to produce a color reaction with ferric chloride.

The chemical nature of the fifth oxygen atom and of the nitrogen present in pantothenic acid becomes apparent from hydrolysis experiments. Pantothenic acid is inactivated by acids and by alkali. From the alkali hydrolysis material, β alanine has been isolated⁴⁴ and identified as β naphthalene sulfo β alanine.⁴⁵ The other part of the molecule is an aliphatic dihydroxy acid which has not been isolated as such. In acid solution, especially upon heating, a lactone⁴⁶ is readily formed, indicating that a hydroxyl group may be in γ - or in δ position to the carboxyl group. The former position is indicated by the previously discussed condensation reaction with acetone and other ketones or aldehydes since the condensation would cause the formation of seven membered rings in case the second hydroxyl group was in δ position.

Pantothenic acid has then the following structural formula



The amino group of the β alanine is bound to the carboxyl group of the dihydroxy acid, forming an acid amide group. Thus, the nitrogen has been accounted for and it has been shown that there are no amino, imino or tertiary amino groups in the molecule. The other hydroxyl group is in α position to the carbonyl group since the acid but not the lactone from the non β alanine portion gives⁴⁷ a positive ferric chloride test⁴⁸ and since carbon monoxide has been obtained⁴⁷ by sulfuric acid decomposition of the dihydroxy acid. The α, β positions of the two hydroxyl groups are excluded by the failure⁴⁷ of pantothenic acid to react with lead tetra acetate and with periodic acid. The lactone has been obtained by ether extraction

⁴³ D. W. Woolley, H. A. Waisman and C. A. Elvehjem, *J. Am. Chem. Soc.* 61, 947 (1939).

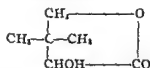
⁴⁴ H. H. Weinstock, H. K. Mitchell, E. F. Pratt and R. J. Williams, *Ibid.* 61, 1471 (1939).

⁴⁵ D. W. Woolley, H. A. Waisman and C. A. Elvehjem, *J. Biol. Chem.* 129, 673 (1939).

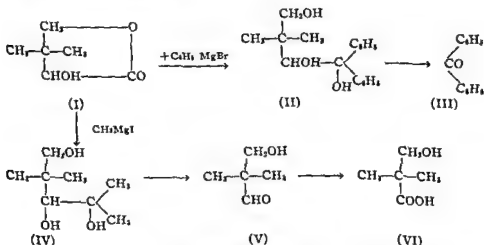
⁴⁶ H. K. Mitchell, H. H. Weinstock, E. L. Snell, S. R. Stanbery and R. J. Williams, *J. Am. Chem. Soc.* 62, 1776 (1940).

⁴⁷ M. A. Berg, *Bull. soc. chim.* (3) 11, 882 (1894).

of acid hydrolyzates as a pure crystalline material^{49 50} and proved to be α hydroxy $\beta \beta$ dimethyl γ butyrolactone⁵⁰



Direct titration⁵¹ of this compound showed the absence of a free carboxyl group but on heating with alkali one mol of alkali was consumed proving the presence of a lactone ring. The lactone contains one active hydrogen atom and one hydroxyl group since a mono acetate was obtained upon acetylation. Oxidation⁵¹ of the lactone with cold alkaline barium hydroxide yielded acetone which indicates the presence of a dimethyl group. This was furthermore shown by a Kuhn Roth determination of CH_3 side chains. Upon reaction with phenyl magnesium bromide the lactone yields a diphenyl carbinol (II) which upon treatment with lead tetra acetate forms benzophenone (III). Methyl magnesium iodide when reacted with the lactone, forms a dihydroxy dimethyl carbinol (IV), which upon oxidation with lead tetra acetate forms the aldehyde (V). The latter on oxidation with alkaline silver hydroxide yields $\alpha \alpha$ dimethyl β hydroxy propionic acid (VI), thus proving the structure of the complete carbon skeleton. By comparing the lactone with synthesized material it was shown to be the laevorotatory form⁵¹



The $\alpha \gamma$ dihydroxy $\beta \beta$ dimethyl butyric acid is dextrorotatory

⁴⁹ D. W. Woolley *Science* 91 245 (1940)

⁵⁰ R. J. Williams and R. T. Major *Ibid* 91 246 (1940)

⁵¹ F. T. Stiller, J. C. Keresztesy and J. F. K. Isten *J. Am. Chem. Soc.* 62 1779 (1940)

7 Synthesis

At a time when the structure of pantothenic acid was unknown, a hemi-synthesis was achieved⁵²⁻⁵³ by condensation of synthetic β alanine ethyl ester with the dihydroxy carboxylic acid isolated from the hydrolysis products of pantothenic acid. The dihydroxy acid was acetylated and converted into the acid chloride for utilization in the condensation. After careful saponification of the condensation product pantothenic acid was obtained.

Since the utilization of an acetylated acid chloride is somewhat difficult and gives low yields, another method has been worked out. This consists in the condensation of an ester of β alanine with the lactone of the dihydroxy acid⁵⁴⁻⁵⁶. Yields up to 50% are obtained by using this procedure.

An improved method, both simple and effective, is the direct condensation⁵⁴ of β alanine (IV) with the lactone (III). This method has the advantage of avoiding the use of esters of β alanine which polymerize on standing⁵⁷ and, furthermore, of yielding pantothenic acid or its calcium salt directly, thus avoiding the saponification procedure after condensation. Approximately a theoretical yield can be obtained. This type of condensation has also been carried out with the dry sodium salt of β alanine⁵⁴⁻⁵⁵⁻⁵⁸⁻⁵⁹ and yields directly the sodium salt of pantothenic acid. The sodium salt can also be obtained⁵⁹ from the ethyl ester of pantothenic acid by hydrolysis with barium hydroxide followed by reacting the obtained barium salt with sodium sulfate. Pantothenic acid has also been synthesized⁶⁰ by condensation of the lactone (III) with β alanine benzyl ester (VI) followed by catalytic hydrogenation of the pantothenic acid benzyl ester (VII) to the free pantothenic acid (V). The synthetically obtained racemic pantothenic acid can be resolved into its components by crystallization of its quinine⁶⁰⁻⁶¹ quinine methohydroxide⁶² or cin-

⁵² D. W. Woolley, H. A. Waisman and C. A. Elvehjem, *J. Am. Chem. Soc.* **61**, 977 (1939).

⁵³ D. W. Woolley, H. A. Waisman and C. A. Elvehjem, *J. Biol. Chem.* **129**, 673 (1939).

⁵⁴ R. J. Williams, H. K. Mitchell, H. H. Weinstock and L. E. Snell, *J. Am. Chem. Soc.* **62**, 1784 (1940).

⁵⁵ T. Reichstein and A. Grüssner, *Helv. Chim. Acta* **23**, 650 (1940).

⁵⁶ R. J. Williams, *Science* **89**, 486 (1939).

⁵⁷ E. Abderhalden and A. Fodor, *Z. physiol. Chem.* **85**, 118 (1913).

⁵⁸ S. H. Babcock and T. H. Jukes, *J. Am. Chem. Soc.* **62**, 1678 (1940).

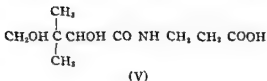
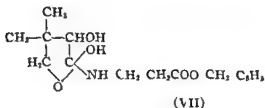
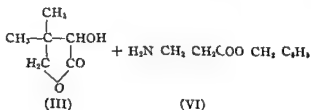
⁵⁹ M. Gätzl, F. Fichter, H. Reich and T. Reichstein, *Helv. Chim. Acta* **24**, 185 (1941).

⁶⁰ R. Kuhn and T. Wieland, *Ber.* **73**, 971, 1134 (1940).

⁶¹ A. Grüssner, M. Gätzl, F. Fichter and T. Reichstein, *Helv. Chim. Acta* **23**, 1276 (1940).

⁶² E. T. Stiller and P. F. Wiley, *J. Am. Chem. Soc.* **63**, 1237 (1941).

chonidine methohydroxide⁶¹ salts. The (+) compound is identical with the naturally occurring pantothenic acid.



The synthesis of the α hydroxy β β dimethyl γ butyrolactone has been carried out as follows.⁶²⁻⁶⁵ Isobutyro aldehyde (I) is condensed⁶⁷ with formaldehyde to give α α dimethyl β hydroxy propionaldehyde (II) which upon condensation with hydrocyanic acid, or better by condensation with potassium cyanide in the presence of calcium chloride⁶⁸ or by reaction of the bisulfite compound of the aldehyde (III) with potassium cyanide, yields racemic α hydroxy β , β dimethyl γ butyrolactone (III).

⁶² E. Glasner, *Monatsh.* 25, 46 (1904).

⁶³ M. Kohn and V. Neustädter, *Ibid.* 39, 293 (1918).

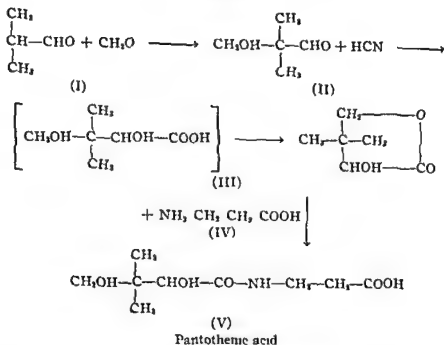
⁶⁴ E. T. Stiller, S. A. Harris, J. Fink, I. Stein, J. C. Kereslasy and K. Folkers, *J. Am. Chem. Soc.* 62, 1785 (1940).

⁶⁵ T. Reichenstein and A. Grusner, *Helv. Chim. Acta* 23, 60 (1940).

⁶⁶ L. Wesely, *Monatsh.* 21, 231 (1900).

⁶⁷ H. E. Carter and L. F. Ney, *J. Am. Chem. Soc.* 63, 312 (1941).

PANTOTHENIC ACID



The natural dextrorotatory pantothenic acid yields upon hydrolysis the (+) α, γ dihydroxy β, β dimethyl butyric acid. The lactone corresponding to this (+) dihydroxy acid is the (–) form.

The racemic, synthetically obtained α hydroxy β, β dimethyl γ butyrolactone can be resolved by conversion into the quinine,⁶⁵ ⁶⁶ quinine methoxide, quinine methoxide or cinchonine methoxide⁶⁹ salt. Thus, the pure (–) α, γ dihydroxy β, β dimethyl butyrolactone can be obtained. The isomeric (+) lactone can be racemized by heating the sodium salt.

β Alanine can be prepared from β halogenated propionic acids and ammonia⁷⁰ or from succinimide by means of hypobromide and potassium hydroxide.⁷¹ Commercially β alanine is produced by adding ammonia to the double bond of acrylic esters⁷ followed by saponification of the ester with barium hydroxide. An excellent alternative method is the catalytic hydrogenation of ethyl cyanoacetate.⁷²

8 Industrial Methods of Preparation

Pantothenic acid is commercially available in the form of its crystalline synthetic calcium or sodium salt prepared according to the previously

⁶⁵ R. T. Major and J. Finkelstein, *J. Am. Chem. Soc.* **63**, 1308 (1941).

⁶⁶ W. H. Metzger, *ibid.* **156**, 30 (1934); F. Mulde, *ibid.* **9**, 1901 (1911).

⁶⁷ S. Hoogwerff, I. W. A. J. Dorj, *Rec. Trav. Chim.* **10**, 4 (1911); H. T. Clark, I. D. Behr,

Org. Syn. Coll. **16**, 1 (1936).

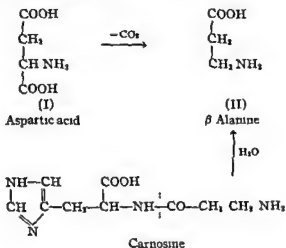
⁶⁸ V. W. D. C. (Ch. Ind.) **19**, 418 (1938); K. M. G. M. Ish, *ibid.* **63**, 220 (1937).

⁶⁹ F. W. G. R. **74**, 2 (1911).

described methods. Pantothenic acid concentrates for example from rice brans, from which most of the other vitamins of the B complex are removed by treatment with fuller's earth are also marketed. Extracts from certain strains of yeast and bacteria are being prepared which contain most members of the vitamin B complex including pantothenic acid. The residues of liver preparations which have been worked up for the antipernicious anemia factor contain considerable amounts of pantothenic acid and are used commercially for the extraction of this vitamin.

9 Biogenesis

The biogenesis of pantothenic acid is not known, but it is reasonable to assume that those organisms which are able to synthesize this vitamin accomplish this by condensation of β alanine with the dihydroxy dimethyl butyric acid or its lactone. The biogenesis of this acid has not been elucidated. β Alanine may arise from a number of different reactions. Thus it has been shown that β alanine (II) is obtained by various bacteria upon decarboxylation of aspartic acid (I)⁷⁴. Certain bacteria are able to hydrolyze carnosine (III) to β alanine⁷⁵.



10 Specificity

Natural pantothenic acid appears to be a single substance⁷⁴ of considerable specificity. Even the optical isomer, the (-) pantothenic acid is

¹¹ D. Ackermann: *Z. physiol. Chem.* 54 1 (1907) 60 482 (1909) *Z. Bot.* 56 87 (1908) A. I. Virtanen and T. Laiho: *Suomen Kemistilehti* B10 2 (1937) *E. symologia* 3 766 (1937) *Nature* 142 674 (1938).

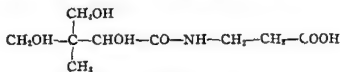
¹⁰ J. H. Muller, *J. Biol. Chem.* **123**, 421 (1938).

^a H. K. Mitchell, E. E. Saelle and R. J. Williams *J. Am. Chem. Soc.* 62 1791 (1940).

inactive in bacterial⁷⁷ and rat assays^{61 73} Both the carboxyl group and the hydroxyl groups are necessary for the physiological action The acetate,⁷⁹ benzoate and diphosphate⁸⁰ of the acid are inactive⁷⁹ The salts of pantothenic acid and certain esters, such as the ethyl ester, are active⁸¹ β Alanine alone may act as a growth stimulant for yeast⁸¹ certain strains of diphtheria bacillus^{82 83} and to a certain extent for rats,^{84 85} but not for chicks It has been shown that organisms which depend upon an external supply of β alanine alone convert this compound into pantothenic acid^{81 83} Other microorganisms, for example lactic acid bacteria,⁸⁶ are unable to utilize only β alanine Some hemolytic bacteria have been found⁸⁷ which require only an external supply of the dihydroxy dimethyl butyric acid part of the pantothenic acid, but not the β alanine part

Chondroitin sulfuric acid has some growth promoting action on rats^{88 89} but its effect even in large doses is slight compared with that of pantothenic acid

A number of compounds, similar in structure to pantothenic acid, have been prepared Of these, the most interesting one is 'hydroxy pantothenic acid' (I) prepared⁵⁹ from β alanine and α hydroxy β methyl β hydroxy methyl butyrolactone This compound possesses striking biological activity which varies, according to the organism used for testing and the assay conditions, between 15% and 25% of pantothenic acid



(I)

Hydroxy pantothenic acid

⁷⁷ E T Stiller S A Harris J Finkelstein J C Keresztesy and L Folkers *J Am Chem Soc* 62 1785 (1940)

⁷⁸ R Kuhn and T Wieland *Ber* 73 971 1134 (1940)

⁷⁹ D W Woolley H A Waitsman O Mickelsen and C A Elvehjem *J Biol Chem* 125 715 (1938)

⁸⁰ D W Woolley *Ibid* 134 461 (1940)

⁸¹ H H Weinstock H K Mitchell C F Pratt and R J Williams *J Am Chem Soc* 61 1421 (1939)

⁸² J H Mueller *Proc Soc Exptl Biol Med* 36 706 (1937) J H Mueller and A W Klotz *J Am Chem Soc* 60 3086 (1938)

⁸³ W C Evans W R C Handley and F C Happold *Brit J Exptl Path* 20 396 (1939)

⁸⁴ M Hoffer and T Reichstein *Nature* 144 74 (1939)

⁸⁵ M M El Sadr H G Hind T F Macrae C F Work B Lythgoe and A R Todd *Ibid* 144 73 (1939)

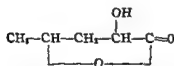
⁸⁶ E E Snell F M Strong and W H Peterson *J Am Chem Soc* 60 2825 (1938) *Biochem J* 31 1789 (1937)

⁸⁷ D W Woolley *J Biol Chem* 130 417 (1939)

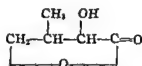
⁸⁸ H E Robinson R E Gray F F Chesley and L A Crandall *J Nutrition* 17, 2-7 (1939)

⁸⁹ H K Mitchell E E Snell and R J Williams *J Am Chem Soc* 62 1791 (1940)

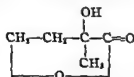
The other compounds tested possess definite, but small activity (less than a fraction of 1% of the activity of pure pantothenic acid) These compounds have been prepared by condensation of β alanine with the following acids or lactones



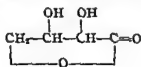
α Hydroxy γ valerolactone^{30 31}



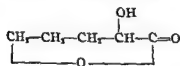
α Hydroxy β methyl γ butyrolactone³⁰



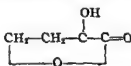
α Hydroxy α methyl γ butyrolactone³⁰



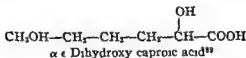
α β Dihydroxy γ butyrolactone³⁰



α Hydroxy δ valerolactone^{30 31 32}



α Hydroxy γ butyrolactone³⁰



The high structural specificity of pantothenic acid is also evident from the failure to obtain active compounds by replacing the β alanine part of the molecule with other amino acids. Thus the analogous compounds obtained from α alanine, from the methyl homolog β amino butyric acid from carboxy β alanine (aspartic acid)³² and from *l* leucine³⁴ proved to be inactive

11 Determination

There are no specific physical or chemical tests known for pantothenic acid. The following biological tests have been proposed and have been used for the determination of various samples of crude preparations

³⁰ H. K. Mitchell, H. H. Weinstock, E. E. Snell, S. R. Stanbery and R. J. Williams *J. Am. Chem. Soc.* 62 1776 (1940)

³¹ T. Reichstein and A. Grüssner *Helv. Chim. Acta* 23 650 (1940)

³² Y. Subbarow and L. Rane *J. Am. Chem. Soc.* 61 1616 (1939)

³³ H. H. Weinstock, E. L. May, A. Arnold and D. Price *J. Biol. Chem.*, 135 343 (1940)

³⁴ R. Kuhn and T. Weland *Ber.* 73 962 (1940)

1 **Chick test** according to Jukes and Lepkovsky^{95 96} In this test, the growth of chicks and the prevention or cure of the specific chick dermatitis is measured

2 **Rat test**^{97 98 99} The increase in weight over a five week period is measured

3 **Bacteria tests** This is a growth test for *Proteus morganii*,^{100 101} lactic acid organisms^{102 103 104 105} and hemolytic streptococci¹⁰⁶ The potency of unknown material is judged by quantitative estimation of the turbidity caused by the growth of the bacteria and by titration of the acid produced during growth

4 **Yeast test.**¹⁰⁷ According to this test, the increase in growth of yeast following the administration of pantothenic acid is determined Using this method, five parts in ten billion parts of culture medium (0 0005 γ per ml) can be determined quantitatively¹⁰⁸

12 Standards

No International Unit of pantothenic acid has been established so far The sodium salt of pantothenic acid has been recommended as standard¹⁰⁹

1 g pantothenic acid = 71 000 Chick Units¹¹⁰
= 50 000 000 Streptobacterium Units¹¹¹

Yeast Unit One Yeast Unit¹⁰⁸ of pantothenic acid is that amount which when tested by the yeast growth method^{107 108} is equivalent to one g of a standard dry rice bran extract This extract is prepared from rice bran with 60% methanol

Ratio Yeast Unit to Chick Unit = 5 1¹¹²

⁹⁵ T Jukes and S Lepkovsky *J Biol Chem* 111 119 (1935) 114 109 117 (1936)

⁹⁶ T H Jukes *Ibid* 117 11 (1937)

⁹⁷ C E Edgar M M El-Sadr and T F Macrae *Biochem J* 32 2200 (1938)

⁹⁸ C E Edgar and T F Macrae *Ibid* 31 886 893 (1937)

⁹⁹ G H Hitchings and Y Subbarow *J Nutrition* 18 285 (1939)

¹⁰⁰ M J Pelczar and J R Porter *Proc Exptl Biol Med* 43 151 (1940)

¹⁰¹ M J Pelczar and J R Porter *J Biol Chem* 139 111 (1941)

¹⁰² E E Snell D Pennington and R J Williams *Proc Am Soc Biol Chem J Biol Chem* 133 XCII (1940)

¹⁰³ C E Snell F M Strong and W H Peterson *Biochem J* 31 1789 (1937) *J Bact* 38 293 (1939)

¹⁰⁴ D Pennington E E Snell and R J Williams *J Biol Chem* 135 213 (1940)

¹⁰⁵ E E Snell and L D Wright *Proc Am Soc Biol Chem* 1941 CXIX

¹⁰⁶ Y Subbarow and L Rane *J Am Chem Soc* 61 1616 (1939)

¹⁰⁷ R J Williams and D H Saunders *Biochem J* 28 1887 (1934) R J Williams E D McAlister and R R Roehm *J Biol Chem* 83 315 (1929)

¹⁰⁸ R J Williams J H Truesdail H H Weinstock E Rohrmann C M Lyman and C H McBurney *J Am Chem Soc* 60 2719 (1938)

¹⁰⁹ M Gätzi Fichter H Reich and T Reichstein *Helv Chim Acta* 24 185 (1941)

¹¹⁰ L W McElroy and H Goss *J Biol Chem* 130 437 (1939)

¹¹¹ R Kuhn and T Wieland *Ber* 73 971 1134 (1940)

¹¹² T H Jukes *J Am Chem Soc* 61 975 (1939)

Chick Unit One Chick Unit¹¹³ of pantothenic acid is defined as one tenth of the amount which will just provide for maximal growth when fed daily to a chick three weeks old in conjugation with a diet free of this vitamin

1 Chick Unit = 14 γ pantothenic acid¹¹⁰

Sbm Unit (Streptobacterium Unit) One Sbm Unit of pantothenic acid has been defined as that amount of the acid which under standardized conditions must be present in one cc of a culture medium in order to achieve maximum cell growth of *Streptobacterium plantarum*¹¹⁴

13 Physiology of Plants and Microorganisms

Plants and microorganisms may be classified with respect to pantothenic acid into those which synthesize this vitamin and those which need an external supply¹¹⁵ The latter group may be subclassified into those which need the entire pantothenic acid molecule for example lactic acid¹¹⁶ and propionic acid¹¹⁷ bacteria streptococci and pneumococci,¹¹⁸ those which need only the β alanine part and those which need only the aliphatic di hydroxy carboxylic acid part (for examples see page 264)

The fact that pantothenic acid plays a role in the growth of plants and microorganisms is undeniable but the extent of that role is undetermined At the present time only a stimulating action of pantothenic acid has been proved for example for pea embryos¹¹⁹ The actual necessity of this compound for plant life especially green plant life, has not been convincingly demonstrated¹²⁰

Green plants synthesize pantothenic acid after they develop their photosynthetic apparatus Yeast synthesizes small amounts but only in the absence of an external supply^{121, 122} that is yeast is a parasitoid On the other hand, yeast synthesizes pantothenic acid *ad libitum* from administered β alanine¹²¹

¹¹³ T H Jukes *J Biol Chem* 117 11 (1937)

¹¹⁴ R Kuhn and T Wieland *Ibid* 73 962 (1940)

¹¹⁵ E J Krauskopf E E Snell and E McCoy *Enzymologia* 7 327 (1939)

¹¹⁶ E E Snell F M Strong and W H Peterson *J Am Chem Soc* 60 2875 (1938) *J Bact* 38, 293 (1939)

¹¹⁷ E J Krauskopf E E Snell and E McCoy *Enzymologia* 7 327 (1939)

¹¹⁸ L Kane and Y Subbarow *J Biol Chem* 134 455 (1940)

¹¹⁹ J Bonner and G Axtman *Proc Natl Acad Sci U S* 23 453 (1937)

¹²⁰ R J Williams and E Rohrer *nn Plant Physiol* 10 509 (1935)

¹²¹ H H Weinstock H K Mitchell E P Pratt and R J Williams *J Am Chem Soc* 61 1421 (1939)

¹²² R J Williams W A Mosher and E Rohrmann *Biochem J* 30 2036 (1936)

14 Animal Physiology

Very little is known about the physiology of pantothenic acid and its metabolism in man and animals. Since pantothenic acid is a vitamin it must be concluded that it is adsorbed from the intestinal tract but it is not known if it is adsorbed as such or after partial or total hydrolysis into the two chemically different parts. It may be assumed that in the intestines pantothenic acid is freed from the protein material to which it is bound in animal tissues although some evidence exists that the bound vitamin is considerably less well utilized than the free vitamin. Blood contains a constant amount of this vitamin and the liver and kidneys are apparently able to store this compound to a certain extent. In tissues from chicks fed a pantothenic acid deficient diet the content of this vitamin was found decidedly lower than normal.¹²² In the spinal cord, brain muscle and blood the differences exceeded 50%, in the liver and kidneys 65%.¹⁴ The blood of men having symptoms of deficiencies of the vitamins B₁, B₂, or nicotinic acid showed also a decrease of 25–50% of the normal level of pantothenic acid.¹⁵ In man, the pantothenic acid content in the serum is increased after injection, but returns to the normal level within one day.¹²⁶ Pantothenic acid is partly destroyed in the organism and is constantly excreted through the urine.¹⁷

In weanling rats kept on a diet deficient in pantothenic acid, the amount of liver fat is markedly less than in control animals kept on a non-deficient diet. Addition of pantothenic acid increases the liver fat.¹²³ The low levels of liver fat can, however, to a certain extent be related to low food intake during the vitamin deficiency.

15 Avitaminosis and Hypovitaminosis

The symptoms of a pantothenic acid deficiency in man are largely unknown and the human need for this vitamin has not been demonstrated. However, since this compound is necessary for rats and chicks, it seems reasonable to assume that it will prove of necessity to man. Indeed a lowering of the pantothenic acid level in blood has been observed in pellagra, beriberi and riboflavin deficiency.¹⁹

¹²² E. E. Snell, D. Pennington and R. J. Williams *J. Biol. Chem.* 133 529 (1940)

¹²³ E. E. Snell, D. Pennington and R. J. Williams *Proc. Am. Soc. Biol. Chem.* 34 XCII (1940)

¹²⁴ S. R. Stanbery, E. E. Snell and T. D. Spies *J. Biol. Chem.* 135 353 (1940)

¹²⁵ T. D. Spies, S. R. Stanbery, R. J. Williams, T. H. Jukes and S. H. Babcock *J. Am. Med. Assoc.* 115 523 (1940)

¹²⁶ D. Pennington, E. E. Snell and R. J. Williams *J. Biol. Chem.* 135 213 (1940)

¹²⁷ R. W. Engel *Proc. Am. Soc. Biol. Chem.* 1941 XXXVII

¹²⁸ S. R. Stanbery, E. E. Snell and T. D. Spies *J. Biol. Chem.* 135 353 (1940)

Chicks on a pantothenic acid deficient diet suffer from a specific dermatosis. Incrustations occur about the eyes, the corners of the mouth and the areas between the toes.¹³⁰ The skin epithelium becomes keratinized and a dry sloughing sets in. Feathering is retarded and the feathers produced are rough. Besides the dermatitis, certain lesions occur in the spinal cord, characterized by degeneration of the myelinated fibers.¹³¹ Chicks suffering from the specific dermatitis often show thymus involution and liver damage (fatty livers).¹³²

Domestic fowls need pantothenic acid for hatchability and reproduction¹³² not, however, for egg production.¹³³ The rate of hatching eggs has been shown to drop from 70% to 3% when the vitamin was removed from the hen's diet.

Pantothenic acid deficiency in young rats¹³⁴,¹³⁵ and mice¹³⁶ causes retardation of growth. A specific symmetrical depigmentation of the fur (nutritional achromotrichia) sets in in rats and mice¹³⁷,¹³⁸,¹³⁹,¹⁴⁰,¹⁴¹ which has also been observed in foxes, especially silver foxes. As the result of this deficiency the fur turns gray.¹³⁹ In albino rats a rustiness of the fur develops¹⁴² and blood caked whiskers have been observed regularly.¹⁴⁰

In rats on a pantothenic acid deficient diet a marked adrenal hemorrhage, atrophy and necrosis have been observed.¹⁴³,¹⁴⁴,¹⁴⁵ No such adrenal changes were found in mice.¹³ Hemorrhages under the skin have also been observed in rats.¹⁴⁰

Another symptom in rats which might be cured by pantothenic acid is the so called 'spectacled eye condition'.¹⁴⁶,¹⁴⁷ (See pages 280 and 477.)

¹³⁰ L. C. Norris and A. T. Ringrose *Science* 71: 643 (1930). A. T. Ringrose, L. C. Norris and G. I. Heus *Poultry Sci.* 10: 166 (1931).

¹³¹ P. H. Phillips and R. W. Engel *J. Nutrition* 18: 277 (1939).

¹³² T. H. Jukes *J. Biol. Chem.* 129: 27 (1939).

¹³³ J. C. Bauernfeld and L. C. Norris *Science* 89: 416 (1939).

¹³⁴ C. E. Edgar, M. M. Elsdon and T. F. Macrae *Biochem. J.* 32: 90 (1938).

¹³⁵ C. H. Hittings and V. Subbarow *J. Nutrition* 18: 265 (1933).

¹³⁶ H. P. Morris and S. W. Lippincott *Proc. Am. Soc. Biol. Chem.* 1941 XCIII.

¹³⁷ P. György, C. E. Poling and V. Subbarow *J. Biol. Chem.* 132: 789 (1940).

¹³⁸ P. György and C. E. Poling *Science* 92: 20 (1940).

¹³⁹ P. György and C. E. Poling *Proc. Soc. Exptl. Biol. Med.* 45: 773 (1940). K. Unna, G. V. Richards and W. I. Sampson *J. Nutrition* 22: 553 (1941).

¹⁴⁰ K. Unna *Proc. Am. Physiol. Soc.* 1941: 285. *J. Nutrition* 20: 565 (1940).

¹⁴¹ M. K. Dimeck and A. Lepp *J. Nutrition* 20: 413 (1940).

¹⁴² H. S. Owens, M. Trautman and E. Woods *Science* 93: 406 (1941).

¹⁴³ F. S. Daft and W. H. Sebrell *U. S. Pub. Health Service Pub. Health Repts.* 54: 2217 (1939).

¹⁴⁴ F. S. Daft, W. H. Sebrell, S. H. Babcock and T. H. Jukes *ibid.* 55: 1333 (1940).

¹⁴⁵ L. L. Ashburn *ibid.* 55: 1337 (1940).

¹⁴⁶ J. J. Oleson, H. R. Bird, C. A. Elvehjem and E. B. Hart *J. Biol. Chem.* 127: 23 (1939).

¹⁴⁷ J. Goldberger and R. D. Lillie *U. S. Pub. Health Service Pub. Health Repts.* 41: 105 (1936). A. Bourque and H. C. Sherman *J. Am. Chem. Soc.* 53: 3501 (1931). H. E. Robinson and R. C. Newton *Abstracts of Biological Chemistry, Am. Chem. Soc., Kansas City, April 13-17 (1936)*. S. Lepkovsky, T. H. Jukes and M. E. Krause *J. Biol. Chem.* 115: 557 (1936).

It has also been postulated that pantothenic acid deficiency causes an earlier aging

(a) Clinical Test Methods

In man and in animals, pantothenic acid can be determined by bacteriological methods either in blood or in urine. For *blood determinations*¹⁴⁸ fresh venous blood is citrated to prevent clotting. The diluted material is then tested according to the bacteria tests as previously described. In normal human beings, the pantothenic acid content of blood ranges from 0.19 to 0.32 γ per cc. and an average of 0.225 γ per cc. has been noted. In patients with vitamin B-complex deficiency a lowered value of 0.05 to 0.09 γ per cc. has been observed. In *urine determinations*,¹⁴⁹ which are best carried out according to the bacteria tests, it is advisable to run check determinations with samples in which the pantothenic acid has been destroyed chemically or to which a known amount of pantothenic acid has been added.

16 Hypervitaminosis

Pantothenic acid is essentially non toxic. At least 100 mg. may be injected intravenously into man without producing any toxic reactions.¹⁵⁰

17 Requirements

Pantothenic acid is probably required by all living matter. It has been shown to stimulate the growth of bacteria (lactic acid bacteria,¹⁵¹ pneumococcus,¹⁵² propionic acid bacteria,¹⁵³ streptococci,¹⁵³ diphtheria bacillus,¹⁵⁴ molds (*aspergillus niger*)), protozoa, fungi, seed plants (alfalfa¹⁵⁵), higher animals (mice, rats,¹⁵⁶ fox, pigs, dogs,¹⁵⁸ birds¹⁵⁹ ¹⁶⁰) and is

¹⁴⁸ S. R. Stanbery, E. F. Snell and T. D. Spies, *J. Biol. Chem.* **135**, 353 (1940).

¹⁴⁹ D. Pennington, E. E. Snell and R. J. Williams, *Ibid.* **135**, 213 (1940).

¹⁵⁰ K. Unna and J. Gresham, *Proc. Soc. Exptl. Biol. Med.* **45**, 311 (1940). T. D. Spies, D. P. Hightower and L. H. Hubbard, *J. Am. Med. Assoc.* **115**, 292 (1940).

¹⁵¹ S. Orla-Jensen, N. C. Otte and A. Snog-Kjaer, *Kgl. Danske Videnskab. Selskab. Skrifte. Naturvidenskab. math. Afdel.* **6**, No. 5, 82 pp. (1936). *Zentr. Bakt. Parasitenk.* (II) **94**, 434 (1936).

¹⁵² L. Rane and Y. Subbarow, *J. Biol. Chem.* **134**, 455 (1940).

¹⁵³ E. J. Krauskopf, E. E. Snell and E. McCoy, *Enzymologia* **7**, 327 (1939).

¹⁵⁴ Y. Subbarow and L. Rane, *J. Am. Chem. Soc.* **61**, 1616 (1939).

¹⁵⁵ C. H. McBurney, W. B. Bollen and R. J. Williams, *Proc. Natl. Acad. Sci. U. S. A.* **21**, 301 (1935).

¹⁵⁶ S. Lepkovsky, T. H. Jukes and M. E. Krause, *J. Biol. Chem.* **115**, 557 (1936).

¹⁵⁷ Y. Subbarow and G. H. Hitchings, *J. Am. Chem. Soc.* **61**, 1615 (1939).

¹⁵⁸ O. Mickelsen, H. A. Waisman and C. A. Elvehjem, *J. Biol. Chem.* **124**, 313 (1938). D. W. Woolley, H. A. Waisman, O. Mickelsen and C. A. Elvehjem, *Ibid.* **125**, 715 (1938). J. M. McKibbin, S. Black and C. A. Elvehjem, *Am. J. Physiol.* **130**, 365 (1940).

¹⁵⁹ J. C. Bauernfeind and L. C. Norris, *Science* **89**, 416 (1939).

¹⁶⁰ T. H. Jukes, *J. Biol. Chem.* **129**, 225 (1939).

probably essential also for human beings. While sheep¹⁸¹ and cattle^{182 183 184} require pantothenic acid, they do not need an external supply since it is synthesized by microorganisms in the rumen.

The amounts required by the various organisms are largely unknown. Yeast needs as growth stimulant 0.008 γ of a purified pantothenic acid per cc of culture medium. In rats a single dose of 800 γ given to depleted animals produced a rapid and marked gain in weight.¹⁸⁵ The optimal daily dose for rats is above 10 γ ¹⁸⁶ and about 100 γ are needed for the cure or prevention of achromotrichia¹⁸⁷ and hemorrhagic adrenal necrosis.^{188 189} For chicks about 500 γ per 100 g of diet appear to be necessary to prevent the typical dermatitis and about 600 γ to insure optimal growth.

¹⁸¹ L. W. McElroy and H. Goss, *J. Biol. Chem.* 130, 437 (1939).

¹⁸² L. W. McElroy and H. Goss, *Ibid.* 133, LXV (1940).

¹⁸³ M. I. Wegner, A. N. Booth, C. A. Elvehjem and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.* 45, 769 (1940).

¹⁸⁴ L. W. McElroy and H. Goss, *J. Nutrition* 21, 405 (1941).

¹⁸⁵ E. T. Stiller, S. A. Harris, J. Finkelstein, J. C. Keresztesy and Y. Folkers, *J. Am. Chem. Soc.* 62, 1785 (1940).

¹⁸⁶ A. Gruasner, M. Glatz, Fichter and T. Reichstein, *Helv. Chim. Acta* 23, 1276 (1940).

¹⁸⁷ P. György and C. E. Poling, *Science* 92, 207 (1940).

¹⁸⁸ P. S. Daft, W. H. Sebrell, S. H. Babcock and T. H. Jukes, *U. S. Pub. Health Service Pub. Health Repts.* 55, 1333 (1940).

¹⁸⁹ L. L. Ashburn, *Ibid.* 55, 1337 (1940).

INOSITOL



INOSITOL

1 Nomenclature

Names

Inositol or more accurately meso or inactive inositol (≠ inositol)

Inosite

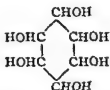
Cyclohexanehexol

Bios I

Mouse antialopecia factor¹

Rat anti spectacled eye factor² (See also pages 269 and 477)

Chemical formula



Empirical formula



2 Chronology

- 1901 WILDIERS³ recognized that yeast needs for optimal growth a special material bios in addition to the known nutrients. This had previously been postulated by LIEBIG in 1869.
- 1924 LUCAS⁴ separated bios into two components bios I and II.
- 1928 EASTCOTT⁵ isolated bios I in the pure form and identified it with the long known inactive inositol.
- 1940 WOOLLEY⁶ produced an alimentary deficiency alopecia in mice and identified the missing factor with inositol.

3 Occurrence

Inositol occurs as a normal cell constituent in practically all plant and animal tissues. In plants the highest amounts are found in the leaves.

¹ D. W. Woolley, *J. Biol. Chem.* 136 113 (1940).

² P. L. Pavek and H. M. B., in *Science* 93 502 (1941).

³ E. Wilder, *La cellulose* 18 313 (1901).

⁴ G. H. W. Lucas, *J. Phys. Chem.* 28 1180 (1924).

⁵ G. V. Eastcott, *ibid.* 32 1094 (1928).

⁶ D. W. Woolley, *Science* 92 384 (1940).

The concentration varies according to the season and reaches its maximum shortly before the time that the fruit ripens. Fruits, especially citrus fruit (lemon, orange, grapefruit), are good sources of inositol.⁷ Cereal grains are also rich sources. Yeast molds and bacteria, at least certain species, contain large amounts of inositol.⁸ Inositol occurs in the animal organism both in the tissues and in the body fluids. Thus inositol has been isolated from skeletal and heart muscle, lungs, kidneys, liver, brain, blood, milk, urine, eggs, etc. Inositol is also found in the eye lens and the optical nerve.⁹

Inositol occurs naturally in a number of different forms. In liver it exists in an alkali labile combination with a large molecule, probably a protein. A combined form also exists in the heart muscle^{10, 11} (for example, that of dogs) and perhaps other types of muscles. Cardiac muscles of sheep, pigs and oxen¹² contain, if any, only very small amounts in the combined form. Some inositol appears to occur in the free state. In plants the majority is present in the form of the hexa phosphate (phytic acid). In the tubercle bacillus¹³ and the soybean inositol is bound in the phosphatide fraction as a glucoside.¹⁴

4 Isolation

Inositol is obtained, for example, from muscles, liver or leaves by hydrolysis with aqueous potassium hydroxide or calcium hydroxide or with concentrated hydrochloric acid,¹⁵ followed by precipitation of large amounts of by products with normal lead acetate. The inositol is then precipitated with basic lead acetate and, after removal of the lead, is precipitated by baryta in alcoholic solution. The free inositol is obtained from the barium precipitate by means of carbon dioxide and is recrystallized, for example, from a minimum amount of water to which alcohol and ether are added to complete the crystallization.¹⁶ Inositol may be prepared from phytin by hydrolysis with formic acid¹⁷ or with calcium hydroxide.

† Inositol can be identified as the hexa acetate m.p. 210° C

⁷ E. K. Nelson and G. L. Keenan *Science* **77** 581 (1933)

⁸ F. Kögl and W. van Hasselt *Z. physiol. Chem.* **242** 43 (1936)

⁹ A. C. Krause and R. Weekers *Arch. Ophthalmol.* **20** 299 (1938)

¹⁰ L. B. Winter *Biochem. J.* **28** 6 (1934)

¹¹ F. Rosenberger *Z. physiol. Chem.* **64** 341 (1910)

¹² L. B. Winter *Biochem. J.* **34** 249 (1940)

¹³ R. J. Anderson and E. G. Roberts *J. Biol. Chem.* **89** 599-611 (1930)

¹⁴ R. J. Anderson, W. C. Lothrop and M. M. Creighton *Ibid.* **125** 299 (1938)

¹⁵ D. W. Woolley *Ibid.* **139** 29 (1941)

¹⁶ Maquenne *Compt. rend.* **104** 225 (1886) *Ann. chim. phys.* [6] **12** 89 (1887) A. Cloetta *Ann.*

99 289 (1856)

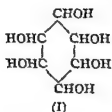
¹⁷ K. Lindenfeld *Biochem. Z.* **272** 284 (1934)

5 Properties

Inositol has a sweet taste. The dihydrate melts at $215-216^{\circ}\text{C}$ while the anhydrous compound melts at $225-226^{\circ}\text{C}$. Inositol is soluble in water (one part in 5.7 parts of water at 24°C), but is insoluble in absolute alcohol and in ether. It crystallizes from acetic acid or from water above 50°C as the water free compound but below 50°C as the dihydrate.

6 Chemistry

Inositol has the formula of a hexahydroxy cyclohexane (I). Theoretically, the latter can exist in nine different cis trans isomers.



Inositol is optically inactive and is often called meso inositol. It has the configuration (II) and forms various esters such as a hexa acetate, a hexa phosphate etc.

7 Industrial Methods of Preparation

Inositol is not a commercially important product. However due to the abundant occurrence of this compound in all sorts of natural materials it should not be difficult to find a cheap raw material which contains relatively high amounts. The recovery of inositol from the steep water of starch factories for example from corn starch plants is of potential commercial value¹⁸. Inositol can also be prepared synthetically by hydrogenation of hexa hydroxy benzene¹⁹.

8 Biogenesis

The mechanism of the inositol formation in plants is not definitely known, but it is generally assumed that inositol is synthesized by aldol type condensations from carbon dioxide and water under the influence of light² or

¹⁸ E. Bartow, W. W. Walker and F. A. Hoglan *Ann. N. Y. Acad. Sci.* 4, 461 (1932)

¹⁹ H. Wieland and R. S. Wharfedale *Ber.* 47, 2082 (1914)

²⁰ R. Kugel *Biochem. Z.* 95, 313 (1919); 97, 21 (1919)

from hexoses by ring closure²¹ Inositol is apparently also synthesized by the chick embryo After about the seventh day of incubation the inositol concentration starts to increase and eventually becomes six times that present in the original egg²²

9 Specificity

Little is known about the specificity of inositol The optical isomers of inositol are inactive The compounds reported to be active in mice are, besides inositol, the naturally occurring phosphoric acid ester, tested as the calcium magnesium salt phytin,²³ inositol hexa acetate and soybean cephalin²⁴ Mytilitol (methyl inositol) is also active Only inositol, and not its esters, is active as a growth factor for yeast²⁴

10 Determination

Specific methods have not been worked out for the determination of inositol especially in natural materials A *chemical method* based on an oxidation with potassium iodo mercurate^{25 26} can be used for determination of about 1-5 mg but is not specific since many polyhydroxy compounds including glycol, mannitol, etc., can be oxidized similarly A *biological method* based on the ability of inositol to cure or prevent alopecia in mice has occasionally been used^{27 28 29} The best method is undoubtedly the *microbiological method* in which the growth of, for example, yeast is measured as a function of the amount of inositol present This procedure has, however, not been perfected

11 Physiology of Plants and Microorganisms

A number of different strains of yeast have been investigated for the growth promoting effect of inositol Some require an external supply of inositol for optimum growth while others show no beneficial effect upon the addition of inositol to the culture medium^{30 31} Similarly, some fung

²¹ F. Michael, H. Rubkopf and F. Suckfull *Ber* 68 1523 (1935)

²² E. E. Snell and E. Quarles *J. Nutrition* 22 483 (1941)

²³ D. W. Woolley *Science* 92 384 (1940)

²⁴ D. W. Woolley *J. Nutrition* 21 Supplement 17 (1941)

²⁵ L. Young *Biochem. J.* 28 1428 1435 (1934)

²⁶ R. A. Gregory *Ibid.* 29 2798 (1935)

²⁷ D. W. Woolley *J. Biol. Chem.* 136 113 (1940)

²⁸ D. W. Woolley *Ibid.* 139 29 (1941)

²⁹ D. W. Woolley *J. Nutrition* 21 Supplement 17 (1941)

³⁰ H. Stantial *Trans. Roy. Soc. Can.* III 26 163 (1932)

³¹ J. B. Lesh, L. A. Underkofer and E. I. Fulmer *J. Am. Chem. Soc.* 60 2505 (1938)

require inositol while others are apparently able to synthesize this compound³²

In higher plants for example, in the tobacco plant, the inositol content increases during growth until maturity is reached. Thereafter the inositol content decreases slowly. Tobacco seeds contain little inositol.³³ Some plants or microorganisms apparently excrete considerable amounts of inositol into the soil since this compound has been isolated in appreciable quantities from agricultural soils.³⁴

There is also the possibility that inositol is used, at least by some species, not only as a growth stimulant but also as a building unit. Thus in caoutchouc a mono- and a dimethyl ether of inositol have been found, the physiological significance of which is not known.

12 Animal Physiology

While little is known about the physiological action of inositol, a few pertinent observations have been made. Inositol occurs in animal tissues to a certain extent in the free form, but also as the phosphoric acid ester for example in the blood of chicken.³⁵ It is thus possible that inositol is phosphorylated in the organism. The possibility however cannot be excluded that the naturally occurring phosphate is directly absorbed.

The mechanism of the inositol action is not known. It has been shown that certain bacteria oxidize one of the hydroxyl groups of inositol to a keto group, but it is not known whether or not such an oxidation occurs in the animal organism. The physiological significance of this oxidation has not been elucidated. It has been claimed that inositol can be oxidized to compounds of unknown composition in muscle, liver, kidney and brain, but in other laboratories no significant effect upon the respiration of the brain tissue of the rat or the rabbit could be observed.³⁶

Inositol markedly increases the peristalsis of the stomach and the small intestine.³⁷ It has been suggested that inositol is the nutritional factor which determines gastrointestinal motility.³⁷

Inositol appears to act as a lipotropic factor. In rats fed a synthetic diet supplemented with the vitamins B₁, B₂, B₆, pantothenic acid, choline and biotin, fatty livers containing large amounts of cholesterol are pro-

³² F. Kögl and N. Fries *Z. physiol. Chem.* 249: 93 (1937).

³³ A. P. Smirnov *State S. R. Inst. Tobacco Makho ka Ind. (U. S. S. R.)* No. 140: 113 (1939).

³⁴ R. K. Yoshida *Soil Sci.* 50: 81 (1940).

³⁵ S. Rapoport *J. Biol. Chem.* 133: 403 (1940).

³⁶ B. C. Guha and N. Das *Current Sci.* 3: 157 (1934); *Z. physiol. Chem.* 231: 157 (1935).

³⁷ L. Young *Proc. Soc. Exptl. Biol. Med.* 35: 507 (1936).

³⁸ G. J. Martin, M. R. Thompson and J. de Carvajal Forero *Am. J. Digestive Dis.* 8: 200 (1941).

duced The development of this special type of fatty liver is prevented by feeding inositol³⁸

13 Avitaminosis

Inositol deficiency symptoms have been noted, especially in young mice^{39, 40} and in rats⁴¹ These rodents require inositol for normal growth and maintenance of hair White mice on an inositol deficient diet become completely hairless over the trunk and severe dermatitis follows In rats, the denudation sets in around the eyes and results in the so called spectacled eye syndrome⁴² Inositol deficiency in rats causes also the development of a special type of fatty liver containing large amounts of cholesterol⁴³

14 Hypervitaminosis

No ill effects have been observed from an intake of inositol but the true toxicity threshold of inositol has not been determined

15 Requirements

The optimum requirements of inositol are largely unknown Deficiency symptoms have been cured in white mice by feeding 10 mg of inositol per 100 g of food⁴⁰ and in rats by feeding a daily dose of 20 mg⁴¹ The requirements of other animals are not known The human requirements have also not been established

³⁸ G Gavin and E W McHenry *J Biol Chem* 139 485 (1941)

³⁹ D W Woolley *Ibid* 136 113 (1940) *Science* 92 384 (1940)

⁴⁰ G J Martin and S Ansbacher *Proc Soc Exptl Biol Med* 48 118 (1941)

⁴¹ P L Pavcek and H M Baum *Am Chem Soc St Louis Meeting April 1941 Div Biol Chem Abstr* p 2

⁴² P L Pavcek and H M Baum *Science* 93 502 (1941) (See however pages 269 and 477)

⁴³ G Gavin and E W McHenry *J Biol Chem* 139 485 (1941)

**PARA-AMINO-
BENZOIC ACID**

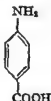
PARA-AMINO-BENZOIC ACID

1 Nomenclature and Survey

Names

p-Amino-benzoic acid
Vitamin B₇^{1a}
B₇ Factor¹
Chromotrichia factor²
Anti gray hair factor
Anticanitic vitamin.²
Trichochromogenic factor^{1b}
Growth factor P for bacteria³

Chemical formula



Empirical formula

C₇H₇O₂N

2 Chronology

1940 Woods and Fildes⁴ observed in *in vitro* experiments an anti sulfanilamide action of *p*-amino benzoic acid Woods⁵ found that yeast extracts contain a factor which counteracts the sulfanilamide activity and suggested that this factor is identical with *p*-amino-benzoic acid SELBIE⁶ was able to inhibit by means of *p*-amino-benzoic acid the action of sulfanilamide in mice infected with *Streptococcus haemolyticus* RUBBO and GILLESPIE⁷ isolated *p*-amino-benzoic acid from yeast FILDEN⁸ concluded that *p*-amino-benzoic acid is an essential metabolite for bacteria

¹ B. Sure *Proc Am Soc. Biol Chem* 1941 CXVX *Science* 94, 167 (1941)

^{1a} G. Lundé and H. Kringstad *N sk Pelsdyrblad* 13 500 (1939)

² S. Ausbacher *Science* 93 164 (1941)

^{2a} L. L. Lachat, *Science* 93 452 (1941) V. H. Engel *Am J Med Sci.* 20* 629 (1941)

^{2b} A. L. Bacharach *Food* 10 219 (1941)

³ H. N. Green *Br J Exptl Path.* 21 38 (1940)

⁴ D. D. Wood and P. Fildes *J Soc. Chem Ind* 59 133 (1940)

⁵ D. D. Woods *Br J Exptl Path.* 21 74 (1940)

⁶ F. R. Selbie *Ibid* 21 90 (1940)

⁷ S. D. Rubbo and J. M. Gillespie *Nature* 146, 838 (1940)

⁸ P. Fildes, *Lancet* 238, 933 (1940)

- 1941 ANSBACHER⁸ found that *p* amino benzoic acid is a vitamin namely a chromo trichia factor for the rat and a growth promoting factor for the chick SIEVE¹⁰ demonstrated that nutritional achromotrichia in man is favorably influenced by *p* amino benzoic acid

3 Occurrence, Isolation and Properties

p Amino benzoic acid is apparently widely distributed^{10a} over the entire plant and animal kingdom but no quantitative data are available *p* Amino benzoic acid is a natural constituent of yeast and has been isolated therefrom as the benzoyl derivative ¹¹ *p* Amino benzoic acid occurs both in a free and combined form ^{11a}

p-Amino benzoic acid is a colorless substance which melts at 186–187° C

4 Determination

A number of chemical and biological methods have been used or suggested for the detection of *p* amino benzoic acid None of these, however, has been sufficiently investigated to determine its degree of specificity

(a) Chemical Method

p Amino benzoic acid in glacial acetic acid yields upon addition of *p* dimethyl amino benzaldehyde in glacial acetic acid a deep yellow color, which can be measured colorimetrically ¹² It has been stated that this method is quite specific and only the *o* and *m* isomers and their derivatives, and aniline and its derivatives, give this test The other vitamins aliphatic amino acids and their aromatic derivatives do not respond to this reagent

(b) Biological Methods

Biological methods for the determination of *p* amino benzoic acid have not been worked out very well Principally, either the cure or prevention of achromotrichia in rats¹³ or the growth of chickens¹⁴ can be used as a criterion for biological determinations A more simple method would be a microbiological test which makes use of the inhibitory action of *p* amino benzoic acid toward the bactericidal effect of sulfonamides It would seem possible also to develop an assay procedure employing a

⁸ S Ansbacher *Science* 93 164 (1941)

¹⁰ B F Sieve *Ibid* 94 257 (1941)

^{10a} N S Dimond *Science* 94 420 (1941) S Wiedling *Science* 94 350 (1941)

¹¹ S D Rubbo and J M Ghespie *Nature* 145 838 (1940)

^{11a} K C Blanchard *J Biol Chem* 140 919 (1941)

¹³ H Tauber and S Laufer *J Am Chem Soc* 63 1488 (1941)

¹⁴ S Ansbacher *Science* 93 164 (1941)

bacterium which needs *p* amino benzoic acid for growth such as *Brucella abortus*¹⁴ or *Streptococcus haemolyticus*¹. Besides growth the production of acid could probably be used as a measure of the *p* amino benzoic acid present when organisms like *Clostridium acetobutylicum*¹⁵ are used as test objects.

5 Physiology

Little is known about the physiological action of *p* amino benzoic acid. Excess amounts given either orally or parenterally are converted in the human and animal organism¹⁷ into the acetate which is secreted through the urine.

In the organism *p* amino benzoic acid counteracts the action of hydroquinone¹⁸ which for example, in the cat¹⁹ and in mice⁶ causes a graying of the fur. The action of sulfanilamide¹ ²² for example in mice infected with *Streptococcus haemolyticus*²³ and the action of sulfapyridine in mice infected with *Pneumococcus*²⁴ are also inhibited by *p* amino benzoic acid.

In vitro para amino benzoic acid modifies melanin formation^{24a} and its possible role in pigmentation processes was suggested^{24b}. It has a pronounced influence on tyrosinase activity,^{24c} inhibits the oxidative destruction of adrenaline^{24d} and appears to act by blocking enzymes^{24e}. Experimental evidence for Woods' hypothesis^{24f} that para amino-benzoic acid and sulfonamides have a common point of attack on some enzyme system or systems is important^{24g}. The sulfonamide inhibition as influenced by para amino benzoic acid was mathematically analyzed^{24h} and found to be of the competitive type as was already experimentally demonstrated⁴.

¹⁴ H. N. Green, *Bull. J. Exptl. Path.* 21: 38 (1940).

¹⁵ F. R. Selbe, *Ibid.* 21: 90 (1940).

¹⁶ S. D. Rubbo and J. M. Gillespie, *Nature* 146: 88 (1940).

¹⁷ A. Ellinger and M. Henkel, *Z. physiol. Chem.* 91: 37 (1914). B. Harrow, F. W. Power and C. P. Sherwin, *Proc. Soc. Exptl. Biol. Med.* 24: 422 (1926-27). K. Bernhard, *Z. physiol. Chem.* 267: 91 (1940).

¹⁸ G. J. Martin and S. Ansbacher, *J. Biol. Chem.* 138: 441 (1941).

¹⁹ H. Oettel, *Arch. exptl. Path. Pharmacol.* 183: 319 (1936).

²⁰ G. J. Martin, *Proc. Am. Assoc. Advance Sci. Pharm. Section*, January 1941.

²¹ D. D. Woods and P. Fildes, *J. Soc. Chem. Ind.* 59: 133 (1940).

²² M. Landy and J. Wyeno, *Proc. Soc. Exptl. Biol. Med.* 46: 59 (1941).

²³ F. R. Selbe, *Bull. J. Exptl. Path.* 21: 90 (1940).

²⁴ M. McCarty, *Proc. Soc. Exptl. Biol. Med.* 46: 133 (1941).

^{24a} G. J. Martin, W. A. Wisniewsky and S. Ansbacher, *Proc. Soc. Exptl. Biol. Med.* 47: 26 (1941).

^{24b} F. L. Pomeroy, *J. Biol. Chem.* 139: 977 (1941).

^{24c} W. A. Wisniewsky, G. J. Martin and S. Ansbacher, *J. Am. Chem. Soc.* 63: 1771 (1941).

^{24d} W. A. Wisniewsky, G. J. Martin, C. T. Tchowski and S. Ansbacher, *J. Am. Chem. Soc.* 64: in press (1942).

^{24e} G. J. Martin, C. T. Tchowski, W. A. Wisniewsky and S. Ansbacher, *Am. J. Physiol.* 127: in press (1942).

^{24f} D. D. Woods, *Brit. J. Exptl. Path.* 21: 74 (1940).

^{24g} J. Kimmig, *Arch. Wechs.* 20: 204 (1941).

^{24h} O. Wys, *Proc. Soc. Exptl. Biol. Med.* 48: 122 (1941).

²⁵ W. A. Wisniewsky, G. J. Martin and S. Ansbacher, *J. Am. Chem. Soc.* 63: 1771 (1941).

6 Avitaminosis

A deficiency in the *p* amino benzoic acid intake causes a graying of the fur in the black or piebald rat, a syndrome which is called nutritional achromotrichia²⁵ A retardation of growth has been observed in chicks on a *p* amino benzoic acid deficient diet²⁵ Nutritional achromotrichia in man has also been cured with *p* amino benzoic acid²⁶ In the female albino rat also a disturbance in the lactation has been noted²⁷ *p* Amino benzoic acid seems to have a therapeutic effect in certain types of asthma,^{27a} possibly because of its protecting or sparing action on adrenaline^{27b}

7 Hypervitaminosis

p-Amino benzoic acid is essentially non toxic²⁸

8 Requirements

The requirements of *p* amino benzoic acid by various species have not been determined In rats 0.75 mg of the acid per day cures nutritional or hydroquinone achromotrichia^{29,30} In mice, 0.25 mg daily prevents depigmentation of the fur^{30a} Chicks have been given²⁹ 30 mg per 100 g of ration, but the lowest optimum amount has not been established In human therapy a dose of 100 mg twice daily has been used successfully³¹

p Amino benzoic acid is required as a growth factor by many bacteria³² such as *Clostridium acetobutylicum*,³³ *Brucella abortus*³⁴ and *Streptococcus haemolyticus*,³⁵ and the addition of this vitamin to all routine culture media has been recommended³⁶

²⁵ S Ansbacher *Science* 93 164 (1941)

²⁶ B F Sieve *Ibid* 94 257 (1941)

²⁷ B Sure *Proc Am Soc Biol Chem* 1941 CXXX *Science* 94 167 (1941)

^{27a} C J Martin and S Ansbacher *Proc Soc Exptl Biol Med* 48 118 (1941)

^{27b} W A Wisansky G J Martin C T Tchiowski and S Ansbacher *J Am Chem Soc* 64 in press (1942)

²⁸ E Strauss F C Lowell and M Finland *J Clin Investigation* 20 189 (1941)

²⁹ S Ansbacher *Science* 93 164 (1941) S Ansbacher and M Landy *Proc Soc Exptl Biol Med* 48 3 (1941) R Richardson and A G Hogan *Proc Soc Exptl Biol Med* 48 459 (1941)

³⁰ G J Martin and S Ansbacher *J Biol Chem* 138 441 (1941)

^{30a} G J Martin and S Ansbacher *Proc Soc Exptl Biol Med* 48 118 (1941)

³¹ B F Sieve *Science* 94 257 (1941)

³² P Fildes *Lancet* 238 955 (1940)

³³ S D Rubbo and J M Gillespie *Nature* 146 838 (1940) J O Lampen and W H Peterson *J Am Chem Soc* 63 2783 (1941)

³⁴ H N Green *Brit J Exptl Path* 21 38 (1940)

³⁵ F R Selbie *Ibid* 21 90 (1940)

³⁶ C A Janeway *J Am Med Assoc* 116 941 (1941)

VITAMIN C—
ASCORBIC ACID

VITAMIN C—ASCORBIC ACID

1 Nomenclature and Survey

Names

- Vitamin C ¹
- Ascorbic acid ²
- Cevitamic acid ³

Historical names

- Hexuronic acid ⁴
- Antiskorbutin (Holst 1912)
- Antiscorbutic vitamin
- Scorbutamin ⁵

Chemical names

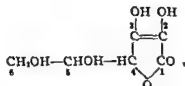
- l Ascorbic acid
- l Threo-3 keto-hexuronic acid lactone
- l Xylo-ascorbic acid
- 3 Keto l gulo-furano-lactone
- l,3 Keto-threo-hexuronic acid lactone
- l-Threo-2 3 4 5 6 pentoxy hexen 2 carboxylic acid lactone

For the purpose of convenience a system of nomenclature has been adopted for compounds related to ascorbic acid in which the term ascorbic acid is preceded by the name of the osone used or theoretically feasible in a synthesis by the osone hydrogen cyanide method (example d gluco-ascorbic acid)

Empirical formula



Structural formula



¹ J C Drummond proposed in 1920 that the compound responsible for the prevention of scurvy be called vitamin C *Biochem J* 14 660 (1920)

² A Szent Györgyi and W N Haworth *Nature* 131 23 (1933)

³ Name introduced by the Council on Pharmacy and Chemistry of the American Medical Association. This organization has now changed the name to ascorbic acid

⁴ A Szent-Györgyi *Biochem J* 22 1357 (1928)

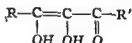
⁵ R. L. Jones *Science* 68, 480 (1928)

Potency

1 g = 20 000 I U

Classification

Ascorbic acid belongs according to its structure and properties to the class of reductones⁸ originally called glucide X and later redoxin (R Wurmser 1927-1934) which are characterized by the constitution



The simplest representative of this class is the enol tartaric dialdehyde⁹



All compounds of this class form reversible oxidation reduction systems

2 Chronology

Scurvy, the typical syndrome of a vitamin C deficiency, has been known for many centuries and occurred epidemically during times of war, crusades, voyages, famines, pestilences, etc (described, for example, by Euricius Cordus in 1534) Fresh vegetables have been known to provide a potent remedy for over three hundred years

1903-1913 BOLLE⁸ BARTENSTEIN¹⁰ and later HOLST and FRÖLICH¹¹ observed that guinea pigs could acquire scurvy just as men could and that the scurvy syndrome is a condition caused by an avitaminosis

1918-1925 ZILVA attempted the concentration of the antiscorbutic substance from lemon obtained almost pure ascorbic acid and established the important basic properties of the vitamin such as the molecular composition and size the resemblance to hexoses the instability toward oxygen especially in alkaline solution etc¹²

1920-1925 AGOPIAN¹³ described methods for the isolation of practically pure ascorbic acid from cabbage

1927 On the basis of the observation that vitamin C solutions immediately after mild oxidation retained their antiscorbutic properties ZILVA¹⁴ concluded that the

⁸ H v Euler and E Klusmann *Arkiv Kemi Mineral Geol* B11 No 12 6 pp (1933)

⁹ H v Euler and C Martius *Swensk Kem Tid* 45 73 (1933) *Arkiv Kemi Mineral Geol* B11 No 14 4 pp (1933) *Ann* 505 773 (1933)

¹⁰ T Reichstein and R Oppenauer *Helv Chim Acta* 16 988 (1933)

¹¹ Bolle *Z diät physik Therap* 6 354 (1903)

¹² L Bartenstein *Jahrb Kinderheilk* 61 6 (1905)

¹³ A Holst *J Hyg* 7 619 (1907) A Holst and T Frölich *Ibid* 7 634 (1907) A Holst and T Frölich *Z Hyg* 72 1 (1912)

¹⁴ A Harden and S S Zilva *Biochem J* 12 259 (1918) S S Zilva *Ibid* 17 416 (1923) 18 185 (1924) 19 589 (1925) S J B Connell and S S Zilva *Ibid* 18 641 (1924)

¹⁵ L A Agopian *F P* 595 537 532 398 26 034 and 27 271

¹⁶ S S Zilva *Biochem J* 21, 689 (1927)

antiscorbutic compound and the reducing factor were closely related although not necessarily identical

- 1928 SZENT GYÖRGY¹⁸ isolated from adrenal glands from oranges and from cabbage a strongly reducing compound the hexuronic acid
- 1932 The identity of vitamin C with SZENT GYÖRGY's hexuronic acid and with ZILVA's 'reducing factor' was discovered by various groups of workers namely by SVIRBELY and SZENT GYÖRGY¹⁹ by WAUGH and KING¹⁷ and by TILLMANS¹⁸ who deduced that vitamin C can be reversibly oxidized and reduced without loss of antiscorbutic efficacy
- 1933 The constitution of vitamin C was established by the combined work of HAWORTH, HIRST and co-workers¹⁹ and of MICHELF and KRAFT²⁰. REICHSTEIN²¹ and HAWORTH²² announced the first successful synthesis of ascorbic acid

3 Occurrence

Vitamin C is widely distributed over the animal and plant kingdoms. It is present in all living plant cells and augmented amounts are found in all actively growing parts of higher plants. Leaves and flowers for example of gladiolus, nettle, hip (hull and shell) and paprika, are especially rich in vitamin C. Excellent sources are the citrus fruits, berries, green vegetables, apples, etc. (see page 324).

Animal tissues contain small but definite amounts of ascorbic acid. Increased amounts are found in various glands, organs, etc. especially those of endocrine functions, for example in liver, hypophysis, corpus luteum, adrenal gland, thymus, etc. Human milk contains considerable amounts of ascorbic acid, blood contains small amounts (for details see pages 325 and 334).

Ascorbic acid occurs in plants predominantly as such. In animal and probably also in plant tissues, ascorbic acid occurs apparently in an equilibrium with its oxidized form, the dehydroascorbic acid.²³ The percentage present of the oxidized and the reduced forms varies considerably according to the tissue and various other physiological factors. In a rabbit for example, the liver was found to contain 27% and muscles

¹⁸ A. Szent György, *Biochem J* 21 1587 (1928)

¹⁹ J. L. Svirbely and A. Szent György, *Nature* 129 576 (1937), *Biochem J* 26 805 (1932)

¹⁷ W. A. Waugh and C. G. King, *Science* 75 357 (1931), *J. Biol. Chem.* 97 375 (1932)

¹⁸ J. Tillmans and P. Hirsch, *Biochem Z* 250 312 (1931)

¹⁹ R. W. Herbert, E. G. V. Percival, R. J. W. Reynolds, F. Smith and E. L. Hirst, *J. Soc. Chem. Ind.* 52 221 481 (1933), E. G. Cox and T. H. Goodwin, *J. Chem. Soc.* 1936 769

²⁰ F. Michelf and K. Kraft, *Z. physiol. Chem.* 222 235 (1933)

²¹ T. Reichstein, *Nature* 132 280 (1933), T. Reichstein, A. Grüssner and R. Oppenauer, *Helv. Chim. Acta* 16 561 1019 (1933)

²² R. G. Ault, D. L. Baird, H. C. Carrington, W. N. Haworth, R. W. Herbert, E. L. Hirst, E. G. V. Percival, F. Smith and M. Stacey, *J. Chem. Soc.* 1933 1419, D. L. Baird, W. N. Haworth, R. W. Herbert, E. L. Hirst, F. Smith and M. Stacey, *Ibid.* 1934 67

50% of the oxidized form. Fresh blood and fresh milk^{23 24 25} contain no dehydro ascorbic acid, but only the reduced form

Besides free ascorbic acid and dehydro ascorbic acid, vitamin C occurs both in animal and in plant tissues in "combined form"^{26 27 28}. The composition of this combined form, which is called "ascorbigen," is unknown (see page 301). It has been shown²⁹ that, for example, livers of fresh water fish contain from 0.5–20% of the total vitamin C content in the combined form, whereas muscle tissue of the same fish contains only 0.1–0.5%. Cow's milk apparently does not contain any combined ascorbic acid, but this form has been found in human milk³⁰. (There is considerable controversy as to whether or not the combined form actually exists,^{30 31} depending upon the technic used for the detection of the combined form.)

4 Isolation

The isolation of vitamin C, which was achieved a number of years before the compound was recognized as a vitamin, is largely dependent upon a quick removal from its solutions. Vitamin C in solution is easily oxidized. This oxidation does not occur to the same extent as long as the vitamin is in its natural environment, which contains protective anti-oxidants (for details see page 327). All isolation procedures must, therefore, be carried out in the absence of oxygen, in the absence of copper,³² which catalyzes the oxidation, and in the absence of light,³³ especially when riboflavin,³⁴ or other fluorescent compounds are present in the raw materials.

Practically, the isolation consists in first preparing a water extract of the starting material (for example, from hips) or pressing the juice out of water-containing materials (for example, of citrus fruits, plant leaves,

²³ E. J. Reedman *Can. Pub. Health J.* 29: 339 (1937)

²⁴ A. Cimmino *Quaderns nutrit.* 5: 239 (1938)

²⁵ C. A. Knight, R. A. Dutcher and N. B. Guerrant *Science* 89: 183 (1939)

²⁶ B. Ahmad *Nature* 136: 797 (1935) E. W. McHenry and M. L. Graham *Ibid.* 135: 871 (1935)

E. J. Reedman and E. W. McHenry *Biochem. J.* 32: 85 (1938) H. Scarborough and C. P. Stewart *Ibid.* 31: 2232 (1937) *Nature* 142: 40 (1938)

²⁷ K. C. Saha *J. Indian Chem. Soc.* 16: 511 (1939) B. C. Guha and P. N. Sen Gupta *Nature* 141: 974 (1938) J. C. Pal and B. C. Guha *J. Indian Chem. Soc.* 16: 481 (1939) P. N. Sen Gupta and B. C. Guha *Ibid.* 16: 496 (1939) B. Ghosh and B. C. Guha *Ibid.* 16: 505 (1939)

²⁸ K. Wachholder and A. Okrent *Z. physiol. Chem.* 264: 254 (1940)

²⁹ K. C. Saha *J. Indian Chem. Soc.* 16: 511 (1939)

³⁰ K. Wachholder and A. Okrent *Z. physiol. Chem.* 264: 254 (1940)

³¹ A. Fujita and T. Ebihara *Biochem. Z.* 301: 229 (1939)

³² A. F. Hess and L. J. Unger *Proc. Soc. Exptl. Biol. Med.* 19: 119 (1921)

³³ C. L. Arcus and S. S. Zilva *Biochem. J.* 34: 61 (1940)

³⁴ D. B. Hand, E. S. Guthrie and P. F. Sharp *Science* 87: 439 (1938) F. C. Hopkins *J. Soc. Chem. Ind.* 56: 934 (1937)

etc) From these aqueous solutions impurities are precipitated with barium acetate or with neutral lead acetate and after filtration, and if necessary after the further addition of lead acetate the vitamin is precipitated as the lead salt by bringing the solution to a pH of about 7.6 (indicator for example bromo thymol blue) with ammonia. The precipitate is filtered or centrifuged and decomposed in water solution with sulfuric or hydrochloric acid by bringing the solution to a pH of about 2. The sulfates or chlorides precipitated thereby are filtered off and washed and coloring matter may now be extracted, for example with *n* butyl alcohol. The combined water solutions are concentrated under vacuum without heating or with only slight heating since temperatures above 60° C decompose the vitamin. The concentrate is purified further by fractional precipitation with organic solvents such as acetone, ether, methanol, ethanol, propanol, butanol, etc. The pure ascorbic acid is then obtained from the filtered solution by distillation of the solvent followed by crystallization of the residue. The vitamin may now be precipitated with acetone or with petroleum ether^{35, 36}. Final crystallization is carried out from methanol.

A modification of this method consists in transforming the ascorbic acid into its acetone derivative (see page 295) and isolating this compound which is then decomposed by water to yield the pure vitamin C.³⁷

By these methods only the ascorbic acid which occurs in the free state is isolated. In order to achieve a better yield the ascorbic acid present in the combined form must first be liberated. This is achieved by gentle heating of a water suspension of the tissue material or of the pressed juice preferably under nitrogen followed by a reduction by means of hydrogen sulfide of the dehydro ascorbic acid originally present and formed during the operations.³⁸

Ascorbigen alone may be isolated from plant material by extraction of the dry material with chloroform, anhydrous ether, etc., followed by water extraction. From juices, for example from cabbage juice, ascorbigen is isolated by adsorption on charcoal followed by elution with a mixture of 30% chloroform and 70% absolute alcohol. Further concentration may be achieved by precipitation of impurities with tungstic acid.³⁹

³⁵ W. A. Waugh, O. A. Bessey and C. G. King, *Proc. Soc. Exptl. Biol. Med.* 30, 1281 (1933).

³⁶ J. L. Svirbely and A. Szent-Györgyi, *Biochem. J.* 27, 279 (1933).

³⁷ E. J. Baumann, *J. Biol. Chem.* 89, 213 (1930); E. J. Baumann and N. Metzger, *Proc. Soc. Exptl. Biol. Med.* 30, 1268 (1933).

³⁸ B. Ghosh and B. C. Guha, *J. Indian Chem. Soc.* 16, 605 (1939).

5 Properties

Vitamin C crystallizes in white, odorless and colorless plates melting at 190–192° C. The vitamin has a somewhat acid taste. Crystallo

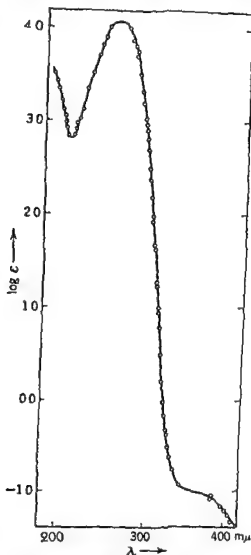


Fig. 14—Absorption spectrum of ascorbic acid in water solution (with an equimolecular amount of KCN) (H. Mohler and H. Lohr)

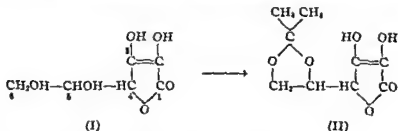
graphic and x ray measurements of the crystallized substance show that the molecule is almost completely flat.³⁹

³⁹ E. G. Cox *Nature* 130 205 (1932). E. G. Cox and T. H. Goodwin *J. Chem. Soc.* 1936 769. E. C. Cox and E. L. Hirst *Nature* 131 402 (1933).

Ascorbic acid is quite soluble in water (one gram dissolves in 3 cc of water), less in alcohols (one gram in 50 cc of absolute alcohol or in about 100 cc of glycerine) and is insoluble in benzene ether chloroform fats, etc. Whereas vitamin C is quite stable in crystalline form it is easily deteriorated in solution especially in the presence of air traces of metals such as copper and iron and light ⁴¹ especially in the presence of riboflavin ⁴¹. The most characteristic property of vitamin C is its strong reducing action in solution and the ease of its oxidation which is catalyzed by some metals especially by copper ⁴² and by silver ⁴³. The oxidation reduction potential of ascorbic acid at pH 4 and at 35° C $E_0' \approx +0.166$ V ⁴⁴ ⁴⁵. In solution vitamin C exhibits acidic properties the dissociation constants being $pK_1 = 4.17$ and $pK_2 = 11.57$. Vitamin C has an optical activity of $[\alpha]_D^{20} = +23^\circ$ in water or $[\alpha]_D^{25} = +48^\circ$ in methanol. Ascorbic acid has a typical ultraviolet absorption spectrum with a maximum at 265 m μ ⁴⁶ ($\log \epsilon = 3.98$) and a small band between 350 and 400 m μ ⁴⁷ ($\log \epsilon = \sim 1$) (Fig. 14) (see also under Determination of vitamin C. Physical methods page 315). The infra red spectrum of vitamin C has also been investigated ⁴⁸.

6 Constitution

Ascorbic acid has the empirical formula $C_6H_8O_6$. It is a monobasic acid, giving well defined salts of the type $C_6H_7O_6M$. Four of the six oxygen atoms belong to four hydroxyl groups as indicated by a determination of active hydrogen atoms. Acetone condenses ⁴⁹ with ascorbic acid (I) with the formation of a mono isopropylidene derivative (II).



⁴¹ C. L. Arcus and S. S. Zilva *Biochem. J.* **34**, 61 (1940).

⁴² P. G. Hopkins *Compt. rend. Acad. Sci. Paris* **22**, 26 (1938).

⁴³ A. F. Hess and L. J. Unger *Proc. Soc. Exptl. Biol. Med.* **19**, 119 (1921).

⁴⁴ F. Schlemm, E. B. Bleyer and H. Cahoon *Biochem. Z.* **254**, 187 (1932).

⁴⁵ E. G. Ball *J. Biol. Chem.* **118**, 219 (1937).

⁴⁶ H. Borsook, H. W. Davenport, C. E. P. Jeffreys and R. C. Warner *Ibid.* **117**, 237 (1937).

⁴⁷ F. P. Bowden and C. P. Snow *Nature* **129**, 720 (1932).

⁴⁸ H. Mohr and H. Lohr *Helv. Chim. Acta* **21**, 485 (1938).

⁴⁹ E. Heintz *Compt. rend.* **208**, 1813 (1939).

⁵⁰ L. V. Vargha *Nature* **130**, 847 (1932).

hydroxyl groups is primary and that another hydroxyl group must be in α position to that primary hydroxyl group

Further insight into the structure of vitamin C was obtained by a number of oxidation reactions. Oxidation of ascorbic acid (I) with one oxygen atom, for example, by means of air,⁶⁰ hydrogen peroxide, ferric chloride, quinones, copper acetate,⁶¹ dichloro phenol indophenol in acid solution, iodine in acid or in neutral solution, and even by irradiation with ultra violet light⁶ yields dehydro ascorbic acid (X) which can be reconverted to ascorbic acid by reducing agents such as hydrogen sulfide and hydroiodic acid. The dehydro ascorbic acid, at the moment of its formation, is not an acid but a neutral compound, a lactone. The lactone ring, in contrast to that of ascorbic acid, readily hydrolyzes in water solution with the formation of a free carboxylic acid group (XI). This behavior shows that ascorbic acid is not a true acid in the sense that the acidic properties are caused by a carboxylic acid group but that the acidity is caused by an enolic hydroxyl group.⁶² The open chain dehydro ascorbic acid cannot be reduced to ascorbic acid by means of hydrogen sulfide alone. If, however, simultaneously with the H_2S treatment a lactone formation is achieved, for example, by evaporation in the presence of hydroiodic acid, the vitamin is formed. The opening of the lactone ring is followed by complex reactions or rearrangements of the molecule, as indicated by changes of the absorption spectrum, optical rotation, etc.⁶³⁻⁶⁵ Further oxidation of ascorbic acid with hydrogen peroxide yields oxalic acid⁶⁶ (XII), which suggests that a keto group is in α position to the carboxyl group. Upon oxidation of the vitamin with sodium hypoiodite in alkaline solution, oxalic acid (XII) and *l* threonic acid (XIII) were obtained almost quantitatively.⁶⁷ The latter acid was identified by methylation (XIV) followed by amidation as trimethyl *l* threonamide (XV) and by oxidation with nitric acid as *d* tartaric acid (XVI) the constitution of which was proved by the formation of dimethoxy-*d* succinamide (XVII). This sequence of oxidation reactions can only be explained when the constitution of the first oxidation product, of dehydro ascorbic acid, is that of an

⁶⁰ I. Antener *Helv Chim Acta* 20 742 (1937)

⁶¹ P. Karrer, H. Salomon, K. Schöpp and R. Morf *Ibid.* 16 181 (1933); P. Karrer, H. Salomon, K. Schöpp and R. Morf *Biochem Z.* 258 4 (1933); P. Karrer, G. Schwarzenbach and K. Schöpp *Helv Chim Acta* 16 302 (1933)

⁶² C. L. Arcus and S. S. Zilva *Biochem J.* 34 61 (1940)

⁶³ E. L. Hirst *J. Soc. Chem. Ind.* 52 221 (1933)

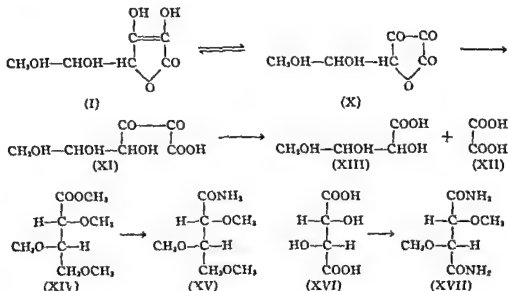
⁶⁴ E. L. Hirst, E. G. V. Percival and F. Smith *Nature* 131 617 (1933); E. L. Hirst, E. G. V. Percival, R. W. Herbert, R. J. W. Reynolds and F. Smith *J. Chem. Soc.* 1933 1270

⁶⁵ H. Borsook, H. W. Davenport, C. E. P. Jeffreys and R. C. Warner *J. Biol. Chem.* 117 1 (1937)

⁶⁶ P. Karrer, G. Schwarzenbach and K. Schöpp *Helv Chim Acta* 16 302 (1933)

⁶⁷ E. L. Hirst *J. Soc. Chem. Ind.* 52 221 (1933)

α,β diketone compound The isolation of *l* threonic acid as a degradation product of ascorbic acid shows that the latter substance is a derivative of *l* gulose This is quite remarkable since practically all naturally occurring hexoses belong to the *d* series



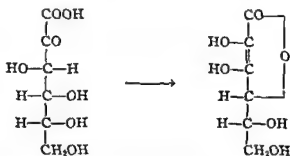
Further degradation reactions were carried out after protecting the four hydroxyl groups. Thus the dimethyl ether of ascorbic acid was esterified with *p* nitro benzoyl chloride yielding a di *p* nitro benzoate which upon oxidation of the double bond gave rise to a neutral ester containing the same number of carbon atoms as the starting material⁸⁸. This proves that the molecule contains a straight chain of six carbon atoms and that the double bond is situated in a ring, or more specifically, in a lactone ring. By saponification of the reaction product, oxalic acid and *l* threonic acid are obtained which shows that the position of the double bond is between carbon atoms 2 and 3 since from the six carbon atom-containing molecule a two- and a four carbon atom containing substance are obtained.

The nature of the ring present in the vitamin C molecule was elucidated by a degradation reaction⁸⁹ very similar to the one just described. Ascorbic acid was methylated with diazomethane to the dimethyl ether (IV), which was further methylated by means of methyl iodide and silver oxide. The tetramethyl ether (XVIII) obtained yielded upon ozonization a

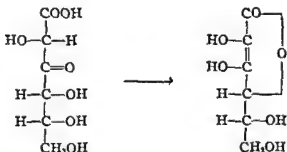
⁸⁸ F. M. Michel and K. Kraft, *Z. physiol. Chem.* 215, 215 (1933).

⁸⁹ E. L. Hart, E. G. V. Percival and F. Smith, *Nat.* 131, 617 (1933); R. W. Herbert, E. L. Hart, E. G. V. Percival, R. J. W. Reynolds and F. Smith, *J. Chem. Soc.* 1933, 1270.

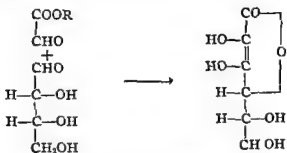
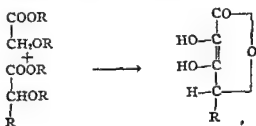
1 By Isomerization and Lactonization of 2-Keto-hexonic Acids



2 By Isomerization and Lactonization of 3-Keto-hexonic Acids



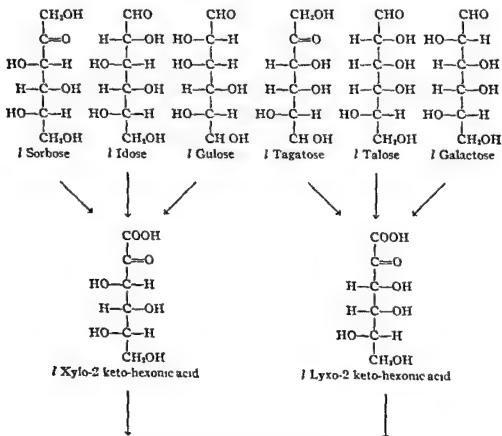
3 By a Benzoin Type Condensation of Two Aldehydes of Lower Molecular Weight

4 By Ester Condensation of α -Oxy-acids

The various methods applicable for the synthesis of ascorbic acid according to the four outlined schemes will be shown in the following paragraphs

(a) *Synthesis of Ascorbic Acid by Isomerization and Lactonization of 2 Keto hexonic Acids*

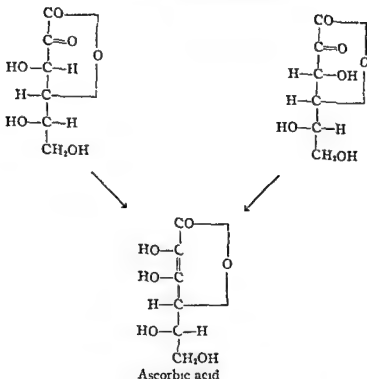
The synthesis of ascorbic acid according to this method¹⁸ is characterized by using a 2 keto hexonic acid as intermediate which may also be called 5 keto hexuronic acid or hexuronic acid. Since such an acid contains one more asymmetrical carbon atom than ascorbic acid namely, the carbon atom 3 there are two different 2 keto hexonic acids which might be converted into ascorbic acid as 1 which have in common the stereochemical configuration at carbon atoms 4 and 5. These two compounds are called



(Formula continued on following page)

¹⁸ T. Reichstein and A. Grünner *Helv. Chim. Acta* 17: 311 (1934)

VITAMIN C—ASCORBIC ACID



l xylo 2 keto hexonic acid and *l* lyxo 2 keto hexonic acid according to the specific configuration of the pentoses to which the hexonic acids correspond. Each of these two 2 keto hexonic acids may in turn be derived from three different hexoses, namely, two aldoses and one ketose. Thus, *l* idose, *l* gulose and *l* sorbose may yield *l* xylo-2 keto hexonic acid which is also called 2 keto *l* gulonic acid, and *l* talose, *l* galactose and *l* tagatose may yield *l* lyxo 2 keto hexonic acid. Of these six hexoses, only *l* sorbose is readily accessible. Besides this aldose also *l* gulose has been used for synthesizing vitamin C.

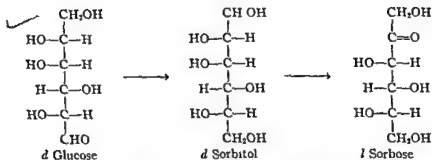
l Sorbose is obtained from *d* sorbitol which occurs in nature and can for example, be isolated from the fruits of mountain ash (*Sorbus aucuparia*). This hexitol is also obtained by catalytic hydrogenation of *d* glucose,⁷⁷ a process which is carried out technically on a large scale.

The *d* sorbitol is converted into *l* sorbose by bacterial oxidation according to Bertrand.⁷⁸ A number of bacteria are able to accomplish this reaction. In practice acetic acid bacteria, are used.⁷⁹

⁷⁷ W. Ipatiev *Ber* 45 3725 (1912) W. E. Cake *J Am Chem Soc* 44 859 (1922) L. W. Floyd R. Connor and H. Adkins *Ibid* 54 1651 (1932) F. P. 694 424 I. G. Farbenindustrie G. P. 544 666

⁷⁸ G. Bertrand *Bull soc chim* [3] 15 677 (1896) *Ann chim* [8] 3 183 227 (1904) H. Schlu bach and J. Vorwerk *Ber* 66 1251 (1933)

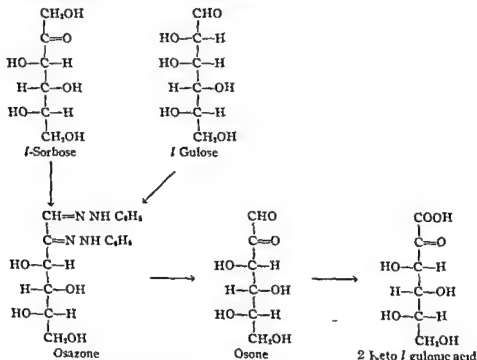
⁷⁹ P. A. Wells, L. B. Lockwood, J. J. Stubbs, N. Forges and E. A. Gastrock *Ind Eng Chem* 31 1425 (1939)



l Gulose which can also be used as starting material for the synthesis of ascorbic acid, is obtained by oxidation of starch or of *d* glucose to *d* saccharic acid, the lactone of which is reduced to *l* gulonic acid. The lactone of the latter compound yields *l* gulose upon reduction.⁸⁰

The oxidation of the hexoses to the 2 keto hexonic acid namely, 2 keto-*l* gulonic acid can be carried out by a number of different methods

(1) *Via the Osone* *l* Sorbose⁸¹ or *l* gulose⁸² is converted into the phenyl hydrazone which in turn is reacted with benzaldehyde to yield the



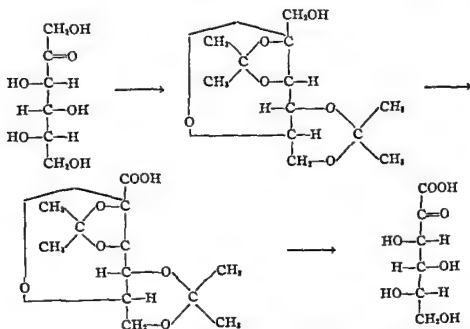
⁸⁰ P P T Sah Ber 69 158 (1936)

⁸¹ P Micheel and K Kraft, Naturwissenschaften 22 205 (1934) P Micheel and W Lohmann Z. physiol Chem 225 13 (1934)

⁸² P P T Sah Ber 69 158 (1936)

corresponding osone This compound must be carefully purified in order to give finally an ascorbic acid which crystallizes well By careful oxidation with bromine⁸³ in aqueous solution, the osone is converted into the 2 keto *l* gulonic acid

(2) *Via the Diacetone sorbose*⁸⁴ *l* Sorbose is condensed with acetone in order to protect all groups with the exception of the primary hydroxyl group in 1 position The main reaction product is sorbose diacetone of the structure indicated below and as a by product a mono acetone sorbose is obtained The latter compound is easily separated from the diacetone derivative since only the di isopropylidene compound is soluble in typical organic solvents, such as ether The mono acetone derivative can be converted into the diacetone compound by condensation with acetone The diacetone sorbose is oxidized by means of permanganate in alkali to the 2 3,4 6 diacetone derivative of 2 keto *l* gulonic acid The free keto acid is obtained by warming the acetone compound in water This last reaction can be carried out in a yield of 82% As a by product small amounts of ascorbic acid are formed



Instead of acetone, other ketones or aldehydes, for example, methyl ethyl ketone, benzaldehyde, etc., and especially cyclic ketones, for example, cyclo hexanone may be used to protect the sorbose

(3) *By direct Oxidation* Sorbose can be oxidized directly to 2 keto

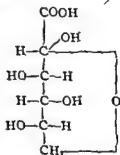
⁸³ C. Neuberger and T. Kitasato *Biochem Z* 183 485 (1927)

⁸⁴ T. Reichstein and A. Grüssner *Helv Chim Acta* 17, 311 (1934)

gulonic acid since the 1 hydroxyl group is especially sensitive to oxidation. This oxidation may be carried out by means of nitric acid⁸⁵ or by catalytic oxidation⁸⁶.

The 2 keto hexonic acids may also be obtained from the corresponding aldonic acids. Thus 1 gulonic acid can be selectively oxidized to 2 keto 1 gulonic by means of chromic acid⁸⁷ or by chlorates in the presence of a vanadium catalyst⁸⁸.

The last step of the synthesis of vitamin C is the conversion of the 2 keto gulonic acid into ascorbic acid by lactonization. This reaction does not occur voluntarily, probably because the keto acid exists mainly in the form of the stable lactol.



This lactonization, can, however, be accomplished by a number of different reaction conditions.

1 From the free keto acid by heating in neutral acid or alkaline solution⁸⁹. The best yields are obtained in acid solution⁹⁰. The double bond produced by enolization of the keto group gives rise to a *cis* and a *trans* compound. Only the *cis* form is able to undergo lactonization the *trans* form is either reconverted into the keto form or undergoes conversion into compounds different from vitamin C.

2 From esters of the keto acid by the action of sodium alkoxides⁹¹ sodium bicarbonate⁹² sodium acetate⁹³ calcium carbonate⁹⁴ etc. The reaction is said to occur also in acid solution⁹¹. The esters of the keto acid are obtained by esterification of the free acid for example with alcohols and mineral acids or with diazo alkanes.

⁸⁵ W. N. Haworth *Nature* 134 724 (1934) B P 443 901

⁸⁶ O. Dalmier and L. Heyns U S P 2 189 778

⁸⁷ R. Iasternack and I. P. Regna U S P 2 153 311

⁸⁸ R. Iasternack and I. P. Regna U S P 2 183 777

⁸⁹ Swiss P 187 933 187 934 180 810 and 183 804

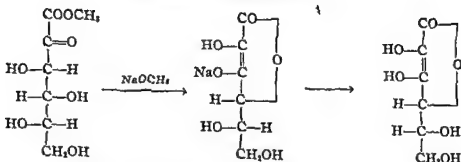
⁹⁰ T. Reichstein and A. Grüssner *Helv. Chim. Acta* 17 311 (1934)

⁹¹ Swiss P 174 208

⁹² Swiss P 187 932

⁹³ Swiss P 180 810

⁹⁴ Swiss P 183 800 183 802 and 183 803



3 From the diacetone keto acid by heating in the presence of diluted mineral acids⁹³

(b) *Synthesis of Ascorbic Acid by Isomerization and Lactonization of 3 Keto hexonic Acids Obtained by the Ozone Hydrogen Cyanide Method*^{95 97 99}

The synthesis of ascorbic acid according to this method involves the utilization of a 3-keto hexonic acid, which also may be called 4 keto hexuronic acid. This acid can practically only be obtained by building up the molecule from a compound of five carbon atoms. This is achieved by the addition of hydrogen cyanide to the osone (IV). Since specific stereochemical configurations are required for the carbon atoms 4 and 5 of ascorbic acid, there is only one pentosone (IV) with the same configuration. Theoretically, this pentosone can be obtained from three pentoses, namely, two aldoses, *l*yxose and *l*xylose, and one ketose. The latter one is still unknown.

Actually, the osone (IV) has been prepared from both aldoses, *l*yxose and *l*xylose. *l*Xylose occurs in nature as part of hemicellulose and can be obtained from soft wood materials, for example, from corncobs, elder pith, beeches, sawdust, shells of coconuts, etc., by acid hydrolysis. It can also be prepared from rice starch or from glucose by oxidation to *d* saccharic acid, reduction to *l* gulonic acid lactone and degradation of the latter compound to *l*xylose. Perhaps the easiest method is to convert *d* sorbitol, which is technically prepared from *d* glucose, into diethylidene sorbitol⁹⁹ by means of paraldehyde or into mono benzal sorbitol¹⁰⁰ by

⁹³ G. P. 641 639 recommends the use of hydrochloric acid whereas Swed. P. 88 094 prefers the use of sulfuric acid.

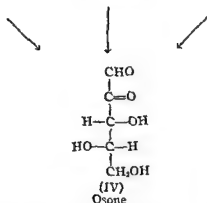
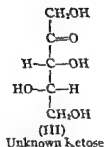
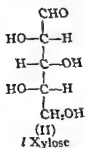
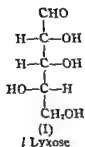
⁹⁵ T. Reichstein, *Nature* 132 280 (1933); T. Reichstein, A. Grüssner and R. Oppenauer, *Helv. Chim. Acta* 16 661 1019 (1933).

⁹⁷ T. Reichstein, A. Grüssner and R. Oppenauer, *Helv. Chim. Acta* 17 510 (1934).

⁹⁹ R. G. Ault, D. K. Baird, H. C. Carrington, W. N. Haworth, R. W. Herbert, E. L. Hirst, E. G. V. Percival, F. Smith and M. Stacey, *J. Chem. Soc.* 1933 1419; D. K. Baird, W. N. Haworth, R. W. Herbert, E. L. Hirst, F. Smith and M. Stacey, *Ibid.* 1934 62.

¹⁰⁰ Hoffman, La Roche, G. P. 627 249; H. Appel, *J. Chem. Soc.* 1935 425.

¹⁰¹ L. v. Varga, *Ber.* 68 18 (1935).



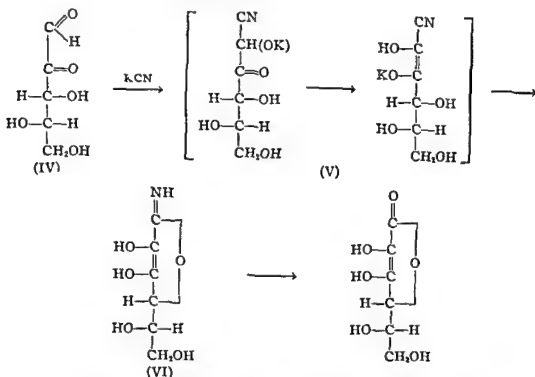
means of benzaldehyde followed by oxidation to di ethylidene *l* xylose or benzal *l* xylose respectively and hydrolysis to the free *l* xylose

The preparation of *l* lyxose is more complicated *d* Galactose (from pectins) is converted into the 1 2 3 4 diacetone derivative and oxidized to 1 2 3 4 diacetone *d* galacturonic acid and hydrolyzed to *d* galacturonic acid By reduction of the aldehyde group of the latter compound *l* galactonic acid is obtained which is converted into the acid amide *l* galactonamide and finally into *l* lyxose

The oxidation of the pentoses to the osone which is called *l* xylosone is accomplished by either preparing first the osazone which is decomposed by the aid of benzaldehyde or by direct oxidation with hydrogen peroxide and ferrous sulfate as catalyst If the osazone is prepared as an intermediate it is not necessary to start with pure crystalline pentoses

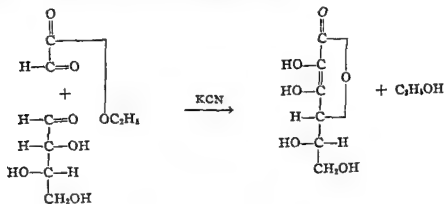
For the conversion of *l* xylosone into ascorbic acid hydrogen cyanide is added to the osone yielding the nitrile (V) which apparently enolizes and lactonizes immediately to form the cyclic imino compound (VI) since it gives none of the reactions for a nitrile group The use of potassium cyanide in the presence of calcium chloride instead of hydrogen cyanide shortens the reaction time from days to hours The cyclic imino-compound (VI) can be isolated The isolation however, is not necessary

By acid hydrolysis *l* ascorbic acid is obtained, the imino group being eliminated as an ammonium salt

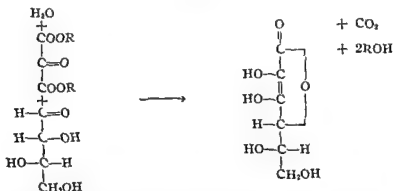


(c) *Synthesis of Ascorbic Acid by Condensation of Ethyl glyoxylate with l Threose*¹⁰¹

According to this method ascorbic acid is synthesized by a benzoin type condensation from ethyl glyoxylate and *l* threose in alkaline solution. Practically, the acetylated cyano hydrine of *l* threose is used which in one operation yields ascorbic acid

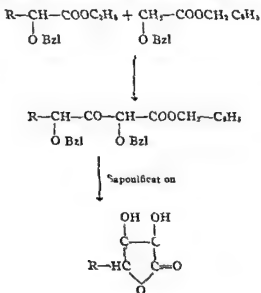


Instead of the ethyl glyoxylate ethyl mesoxylate may also be used with the same effect ¹⁰²



(d) *Synthesis of Ascorbic Acid by Ester Condensation of Benzoyl glycolic Acid with α Oxy acids*¹⁰³

This method is best illustrated by the following formulas



8 Industrial Methods of Preparation

Vitamin C is commercially available in the crystalline form, made either by synthesis or by extraction from natural sources. Since very

¹⁰² B. Helfferich, O. P. 683,954

¹⁰³ F. M. Cebel and H. Haarhoff, Ann. 545, 28 (1940)

economic methods have been worked out for the synthetic route, using the 2 keto hexonic acid as intermediate, the isolation procedure from natural sources becomes less and less attractive. The purity of commercial ascorbic acid is about 99%.

In the canning industry special methods have been developed for essentially maintaining the natural vitamin C content of foods. For example, the water used to cook vegetables is boiled before the plant material is added in order to avoid oxidation by the air dissolved in the water.¹⁰⁴ The boiling process itself is carried out anaerobically.¹⁰⁴ Vegetables are stored in refrigerators where the loss of vitamin C is slow, whereas at room temperature most plant materials lose their vitamin C content rapidly. The freezing method for preserving food causes no essential loss of vitamin C provided the material is consumed immediately after defrosting.

9 Biogenesis

The mechanism of the ascorbic acid formation in plants and in animals is largely unknown. It seems conceivable that ascorbic acid may be produced by transformation of sugar acids of related structure such as glucuronic acid or galacturonic acid, or by total synthesis. In favor of the latter hypothesis is the observation that volatile constituents of plant and animal unsaponifiable matter, lipid in nature, can serve as the precursor of ascorbic acid in the body of the rat.¹⁰ Furthermore, it has been found that the vitamin C content of livers and intestines of rats which had been subjected to extreme periods of inanition did not change significantly. This suggests that the precursor of ascorbic acid was probably of endogenous origin and independent of carbohydrate intake.¹⁰⁸

In favor of the hypothesis that vitamin C is formed by chemical transformation of compounds of related structure is the observation that dextrose apparently increases the ascorbic acid content of slices of intestinal tissue,¹⁰⁷ but not of tissue slices from liver, spleen, stomach and brain, when the vitamin is determined by iodine titration. Among many sugars investigated, mannose causes a greater rise of ascorbic acid than any other sugar investigated, in plant¹⁰⁸ and animal¹⁰⁹ tissue in *in vitro* and *in vivo* experiments. Also *l*-sorbose has been found active as precursor of vita-

¹⁰⁴ E. F. Kohman, W. H. Eddy and C. Z. Gurin *Ind Eng Chem* 23 1064 (1931) 25 682 (1933)

¹⁰⁵ R. R. Musulin, R. H. Tully, H. E. Longenecker and C. G. King *J Biol Chem* 129 437 (1939)

¹⁰⁶ C. Mentzer and G. Urbain *Compt rend soc biol* 128 270 (1938)

¹⁰⁷ F. Widenbauer and K. Koschorreck *Biochem Z* 291 206 (1937)

¹⁰⁸ S. N. Ray *Biochem J* 28 996 (1934)

¹⁰⁹ B. C. Guha and A. R. Ghosh *Nature* 134 739 (1934) 135 234 871 (1935) 138 844 (1936)

min C¹¹⁰ These findings, however, could not be confirmed¹¹¹ It has recently been postulated¹¹² that the presence of traces of manganese is necessary for the successful synthesis of ascorbic acid from mannose and to a lesser extent from galactose and glucose in plant and in animal tissues especially in the liver Also the inability of man and guinea pigs to produce vitamin C has been discussed in view of the low Mn concentration of their tissues¹¹³ It has furthermore been shown that whereas the optimum Mn concentration in *in vitro* and *in vivo* experiments with rat liver tissue is about 0.001-0.005% the concentration of manganese necessary for a successful synthesis of ascorbic acid in *in vitro* and *in vivo* experiments with guinea pig liver tissue is much higher¹¹⁴

A correlation of available manganese with ascorbic acid production has also been found in plants The ascorbic acid content of tomatoes from plants grown on soils low in manganese is considerably lower than from plants grown on soils with higher manganese content In pot cultures the application of one gram of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ in a localized area to 15 000 g of Sassafra sandy loam soil increased the ascorbic acid content in tomato pulp from 142 to 243 mg per liter¹¹⁴

The various observations of increased ascorbic acid excretion following the administration of various chemicals appear to result from a slightly different chemical mechanism¹¹⁵ Particularly effective are terpene like cyclic ketones for example *l* and *d* carvone *d* l piperitone, isophorone α and β ionone pulegone, thujone camphor and nerolidol and somewhat less active are diisobutyl ketone, dipropyl ketone and dimethyl acetyl carbinol These results indicate that a stimulation of the normal ascorbic acid synthesis occurs in order to detoxify compounds which are foreign to the tissues The increase of ascorbic acid content of rat adrenals after feeding 3 hydroxy acetonyl acetone may probably be explained similarly¹¹⁶

For the site of ascorbic acid formation in plants and for the influence of light upon the synthesis see page 324

10 Specificity

The antiscorbutic activity of vitamin C is quite specific A number of salts are active for example the sodium copper manganese and iron

¹¹⁰ G v Szatmari *Biochem Z* 295 369 (1938)

¹¹¹ A Scheunert and M Schulich *Z physiol Chem* 246 972 (1937) J R Hawthorn and D C Harrison *Biochem J* 31 1061 (1937)

¹¹² M N Rudra *Nature* 141 203 (1938) 143 811 (1939) *Biochem Z* 301 938 (1939)

¹¹³ M N Rudra *Nat* 144 868 (1939)

¹¹⁴ J B Hester *Science* 93, 401 (1941)

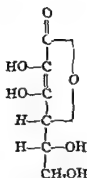
¹¹⁵ H E Longenecker R R Musulin R H Tully and C G King *J Biol Chem* 129 443 (1939)

¹¹⁶ J Mosonyi *Z physiol Chem* 230 940 (1934)

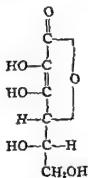
salts, and salts of organic amines, for example, of mono ethanol amine and of quinine. None of the simple derivatives of ascorbic acid for example the acetone derivative,¹¹⁷ the dimethyl ether, the dihydro compound or the imino ascorbic acid, are active. It is important to note however, that the oxidized form, the dehydro ascorbic acid, in lactone form has the same activity as the non oxidized form. The open chain dehydro ascorbic acid, on the other hand, is completely inactive, whereas the methyl 2 keto *l* gulonate, but not the free acid, is active,¹¹⁸ presumably because the organism is able to convert this compound into ascorbic acid.

Practically all the theoretically possible stereoisomers and simple homologs of ascorbic acid have been prepared and tested for antiscorbutic activity. From the results of these experiments it is concluded that for antiscorbutic activity a *d* configuration is necessary at carbon atom 4, that a side chain must be attached to this carbon atom, and that in the side chain a hydroxyl group is necessary in 5 position. All hydroxyl groups must be free.

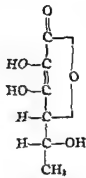
The number of active compounds is therefore, quite limited. They are *d* arabo ascorbic acid^{119 120 121 12} (iso ascorbic acid) (about $\frac{1}{20}$ of the activity of ascorbic acid), 6 desoxy *l* ascorbic acid¹²³ (about $\frac{1}{2}$ of the activity of ascorbic acid), *l* rhamno ascorbic acid¹²⁴ (about $\frac{1}{4}$), *l* gluco ascorbic acid¹²⁵ (about $\frac{1}{40}$), *l* fuco ascorbic acid¹²⁵ (about $\frac{1}{50}$) and *d* gluco



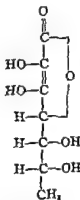
l Ascorbic acid



d Arabo-ascorbic acid



6 Desoxy *l* ascorbic acid



l Rhamno-ascorbic acid

¹¹⁷ F. Michael and T. Moll *Z. physiol. Chem.* 219: 253 (1933).

¹¹⁸ T. Reichstein and V. Demole *Festschrift für F. C. Borell* Basel 1936.

¹¹⁹ T. Reichstein, A. Grüssner and R. Oppenauer *Helv. Chim. Acta* 17: 510 (1934).

¹²⁰ H. Ohle, H. Erbach and H. Carla *Ber.* 67: 324 (1934).

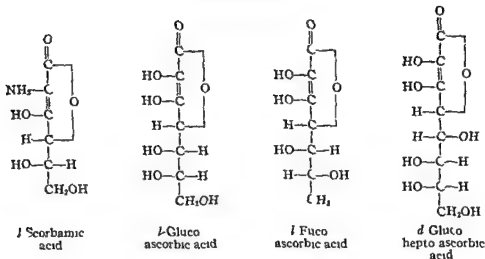
¹²¹ K. Maurer and B. Schiedt *Ibid.* 66: 1054 (1933); 67: 1239 (1934).

¹²² O. Dalmer and T. Moll *Z. physiol. Chem.* 222: 116 (1933).

¹²³ H. Müller and T. Reichstein *Helv. Chim. Acta* 21: 273 (1938).

¹²⁴ T. Reichstein, L. Schwarz and A. Grüssner *Ibid.* 18: 350 (1935).

¹²⁵ T. Reichstein and V. Demole *Festschrift für F. C. Borell* Basel 1936.



hepto ascorbic acid¹²⁶ (about 1/100) *l* Scorbamic acid has also been reported¹²⁶ to show antiscorbutic activity. Of all the compounds *l* ascorbic is the most potent.

Vitamin C is the only naturally occurring antiscorbutic compound. However, another substance has been isolated from adrenal cortex¹²⁷ of beef, which is said to show faint antiscorbutic activity and which possesses the reducing and general chemical properties of ascorbic acid. The composition was found to be C 25.86%, H 5.97%, N 5.97%, P 0.55%. The chemical and physical data of this compound do not permit any conclusion to be drawn as to the purity or the constitution of the compound. It might well be that a form of ascorbigen, a combined ascorbic acid, was isolated. Another possibility would be that ascorbic acid can be chemically bound in a way similar to the vitamins of the B group to complex molecules containing phosphoric acid thus forming part of enzyme systems. Any definite opinion about the existence of a second naturally occurring antiscorbutic substance must be withheld until further experimental data become available.

11 Determination

(a) Physical Methods

Spectroscopical Method The determination of the intensity of the characteristic absorption spectrum in water solution at 265 mμ, for ex

¹²⁶ F. Michael and R. Miltig *Naturwissenschaften* 25 158 (1937); *Z. physiol. Chem.* 247 34 (1937).

¹²⁷ E. Ott, J. Krämer and W. Faust *Z. physiol. Chem.* 243 192 (1936).

ample, before and after destruction of the vitamin, has been advocated.¹²⁸ Since vitamin C in solution is quickly destroyed, the determination must be carried out rapidly. An addition of reducing agents has been recommended. The position of the absorption band is somewhat different in various solvents. In alcohol for example, the maximum is at 245 m μ . The position of the band is, furthermore, a function of the pH. With decreasing pH the maximum shifts toward shorter wave lengths.¹²⁹ The best method of determining ascorbic acid is to use a water solution of the vitamin to which an equimolecular amount of potassium cyanide as stabilizer has been added (Fig. 14 on page 294).^{130 131}

Polarographic Method Determination of the oxidation potential of ascorbic acid in acid solution, for example, in extracts from fruits or vegetables, has given quite satisfactory results.¹³²

(b) Chemical Methods

Chemical methods of determining vitamin C are generally used today replacing almost entirely the biological methods of earlier days. The chemical methods are mostly based on the great reducing ability of ascorbic acid. Since besides ascorbic acid the naturally occurring dehydroascorbic acid also exhibits vitamin C activity, care must be taken to include the non reducing dehydroascorbic acid in determinations of vitamin C in natural products. It should, furthermore, be observed that apparently varying amounts of vitamin C are chemically bound to protein materials, in which combination the ascorbic acid shows no reducing action.

Titration with Iodine The vitamin C content of pure solutions can be determined by titration with 0.01 *N* iodine solution. This method proved to be inadequate for the determination of ascorbic acid in natural products, since they contain other reducing substances besides vitamin C and since the color of such products interferes with the determination of the end point of the iodine titration.

Titration with 2,6-Dichloro-phenol-indophenol The determination of vitamin C by titration with 2,6 dichloro phenol indophenol (see formula p 317) originally suggested by Tillmans¹³³ is the most widely used method today. A great number of different modifications have been

¹²⁸ E. B. Robertson *J. Soc. Chem. Ind.* 53 277 (1934). A. Chevallier and Y. Choron *Compt. rend. soc. biol.* 124 453 (1937).

¹²⁹ B. Starzynski *Bull. Acad. Pol. Sci. Letter. A* 1937 46°.

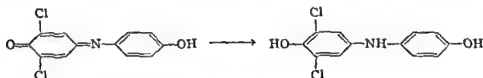
¹³⁰ R. A. Morton *The Application of Absorption Spectra to the Study of Vitamins and Hormones* London 1935.

¹³¹ H. Mobler and H. Lohr *Helv. Chim. Acta* 21 485 (1938).

¹³² K. Schwartz *Z. anal. Chem.* 115 161 (1939). T. Osternd *Tek. Ukeblad* 86 216 (1939).

¹³³ J. Tillmans *Z. Untersuch. Lebensmittel* 54 33 (1937).

proposed to overcome the various insufficiencies resulting from the determination of vitamin C in the presence of other plant or animal materials. The indophenol dye oxidizes besides ascorbic acid for example sulphhydryl compounds¹³⁴ thiosulfate,¹³⁵ pyridinium compounds¹³⁶ (medication) the reduced forms of nicotinic acid derivatives and of riboflavin¹³⁷ etc. In organic and organic ferrous and ferric compounds also interfere with the determination¹³⁸. In beer yeast malt mold etc organic reducing substances are found which react exactly like ascorbic acid toward the indophenol indicator. These compounds are sugar derivatives formed by alkali. Known examples of these are reductive acid¹³⁹ and reductone¹⁴⁰.



2,6 Dichloro phenol indophenol

A certain amount of vitamin C in solution is often present in the dehydro form which is not determined by the indophenol titration. In order to include the dehydro form it is necessary to convert this form first into ascorbic acid. This is carried out by hydrogen sulfide at pH 4-7 followed by an elimination of the excess hydrogen sulfide by blowing nitrogen through the solution. The results obtained by this method, however, are not always trustworthy. The following method has therefore been suggested: (1) to convert the total vitamin C content into the dehydro form for example by passing through Norite or by ascorbic acid oxidase; (2) to determine the amount of reducing substances left; (3) to reduce the dehydro compound with hydrogen sulfide; and (4) to titrate the vitamin C with indophenol¹⁴¹. The difference between the value from the second titration and the value of the first titration gives fairly reliable results of the total amount of ascorbic acid present unless, as in urine, substances are present which are capable of slow reduction with hydrogen sulfide to indophenol reducing substances¹⁴². This difficulty can how

¹³⁴ A. Emmert, *Bochem J* 28 768 (1934)

¹³⁵ M. van Eekelen, *Acta Brevia Neerlandica Physiol Pharmacol Microbiol* 4 137 (1934); M. Heine, *manu Ibid* 6 67 (1936)

¹³⁶ C. F. Cannon and T. McGovern, *Proc Soc Exp Biol Med* 38 267 (1933)

¹³⁷ W. W. Woessner, C. A. Elvehjem and H. A. Schuette, *J Nutrition* 18 619 (1930)

¹³⁸ K. P. Basu and M. C. Nath, *J Indian Chem Soc* 15 133 (1938)

¹³⁹ T. Reichstein and R. Oppenauer, *Helv Chim Acta* 17 390 (1934)

¹⁴⁰ H. K. Nelson and C. A. Browne, *J Am Chem Soc* 51 830 (1929); H. v. Euler and C. Martus, *Ann* 505 73 (1933)

¹⁴¹ A. Emmert and M. van Eekelen, *Bochem J* 28 1151 (1934); 30 75 (1936)

¹⁴² H. Scarborough and C. P. Stewart, *Ibid* 31 2-32 (1937)

ever, be overcome by appropriate use of a photoelectric colorimeter, by following the progress of the titration as a function of time and by extrapolation of the results obtained^{143 144 145} The determination of vitamin C in fruits and vegetables may be carried out by any of these methods with a fair degree of accuracy Since the amount of the dehydro form in the living plant is generally insignificant (it is significant, however, in stored materials), approximate values can be obtained by direct titration if the fresh material is extracted sufficiently rapidly with a strong acid, for example, acetic acid,¹⁴⁶ trichloro acetic acid,¹⁴⁷ metaphosphoric acid,¹⁴⁸ oxalic acid,¹⁴⁹ etc., to diminish the oxidation of vitamin C by the oxidase present in the plant cells and to exclude the titration of glutathione present, and if the titration is performed immediately thereafter

Some other modifications have also been proposed Thus, it has been suggested¹⁴⁰ to extract the tissues in the cold with sulfuric acid or with phosphoric acid, to reduce the extract, for example, with hydrogen sulfide, cadmium, zinc, aluminum, palladium, chromium or titanium at a pH of about 4.5, and to titrate with indophenol before and after an addition of copper sulfate Since the copper ions oxidize preferentially the ascorbic acid, the difference of the two titrations corresponds to the ascorbic acid content

The accuracy of the determination of vitamin C in extracts of animal origin is somewhat limited Liver, for example, may give values up to 20% too high Metaphosphoric acid is particularly useful for the extraction of animal tissues since it also deproteinizes the extracts¹⁵¹ Foreign reducing materials may be removed with lead acetate¹⁵² or with mercuric acetate¹⁵³ The latter, however, also oxidizes ascorbic acid to a considerable extent to dehydro ascorbic acid

Special methods have been worked out to determine the ascorbic acid concentration in blood plasma¹⁵⁴ and in milk,¹⁵⁵ which involve the use of oxalic acid either alone or in mixture with metaphosphoric acid Dehydro ascorbic acid is determined by reduction with hydrogen sulfide

¹⁴³ R. L. Mindlin and A. M. Butler *J Biol Chem* 122 673 (1938)

¹⁴⁴ O. A. Bessey *Ibid* 126 771 (1938)

¹⁴⁵ W. W. Woessner, C. A. Elvehjem and H. A. Schuette *J Nutrition* 18 619 (1939)

¹⁴⁶ R. Strohecker and R. Vaubel *Angew Chem* 49 666 (1936)

¹⁴⁷ T. W. Burch, L. J. Harris and S. Ray *Biochem J* 27 590 (1933)

¹⁴⁸ W. W. Woessner, C. A. Elvehjem and H. A. Schuette *J Nutrition* 18 619 (1939)

¹⁴⁹ E. Willberg *Z Untersuch Lebensm* 76 128 (1938)

¹⁵⁰ M. Ott *Angew Chem* 54 170 (1941)

¹⁵¹ A. Fujita and T. Ebihara *Biochem Z* 290 172 (1936)

¹⁵² V. A. Deviatnin and V. M. Doroshenko *Ibid* 280 118 (1935)

¹⁵³ A. Emmerie and M. Eekelen *Biochem J* 28 1151 (1934) 30 25 (1936)

¹⁵⁴ R. L. Mindlin and A. M. Butler *J Biol Chem* 122 673 (1938)

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All these methods as described include only the determination of ascorbic acid and of dehydro ascorbic acid but not the determination of the combined form ascorbigen although experimental evidence has been presented that the ascorbic acid is liberated from ascorbigen by extraction with metaphosphoric or sulfo salicylic acid^{162 163} In order to determine the total true ascorbic acid content of plant or animal material the vitamin must be liberated from its carrier An aqueous suspension of the material is heated while passing hydrogen sulfide through the solution, and after removal of the excess hydrogen sulfide by carbon dioxide or nitrogen the solution is titrated, for example before and after a treatment with ascorbic acid oxidase The transformation into the dehydro compound by means of the specific enzyme or by some other means such as copper ions appears to be necessary, since during the heating process in the presence of hydrogen sulfide an appreciable amount of reducing compounds other than vitamin C is formed which are capable of being oxidized by the indophenol dye¹⁶⁴

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¹⁵⁶ M Ott *Angew Chem* 51 537 (1938)

¹⁵⁷ K A Evelyn *J Biol Chem* 115 163 (1936) K A Evelyn H T Malloy and C Rosen *Ibid* 126 645 (1938)

¹⁵⁸ T Guthe and K H Nygaard *J Soc Chem Ind* 57 1195 (1938)

¹⁵⁹ R L Mindina and A M Butler *J Biol Chem* 122 673 (1933)

¹⁶⁰ O A Bsey *Ibid* 126 771 (1938)

¹⁶¹ F Sebert *Dissertation* Frankfurt a Main 1931

¹⁶² K Wachholder and A Okrent *Z physiol Chem* 264 254 (1940)

¹⁶³ A Fujita and T Chihara *Biochem Z* 301 29 (1939)

¹⁶⁴ P N Sen Gupta and B C Guha *J Ind an Chem Soc* 16 549 (1939)

¹⁶⁵ H Lund and H Lieck *Natur* 137 784 (1936)

¹⁶⁶ I Gál, *Ibid* 138 799 (1936)

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the sensitivity of the determination of ascorbic acid with indophenol is satisfactory and in questionable cases, for example, in cases of silent turbidity, the results obtained are checked by adding a known amount of ascorbic acid to the solution.

The end point of the titration with the indophenol dye may be determined by visual observation or by the aid of a colorimeter¹⁵⁶ or a photometer^{157 158 159}. The latter method is especially accurate since a decrease in concentration of the dye produced by the addition of a vitamin C containing extract of insufficient concentration to cause complete decoloration of the indicator,¹⁶⁰ can be measured. The titration of very dark solutions may be carried out by extracting the excess dye after the complete oxidation of the vitamin.¹⁶¹

All these methods as described include only the determination of ascorbic acid and of dehydro ascorbic acid, but not the determination of combined form, ascorbigen although experimental evidence has been reported that the ascorbic acid is liberated from ascorbigen by extraction with metaphosphoric or sulfo salicylic acid.^{162 163} In order to determine the total true ascorbic acid content of plant or animal material the vitamin must be liberated from its carrier. An aqueous suspension of the material is treated while passing hydrogen sulfide through the solution, and after removal of the excess hydrogen sulfide by carbon dioxide or nitrogen the solution is titrated for example before and after a treatment with ascorbic oxidase. The transformation into the dehydro compound by means of a specific enzyme or by some other means such as copper ions appears to be necessary, since during the heating process in the presence of hydrogen ions an appreciable amount of reducing compounds other than vitamin C is formed which are capable of being oxidized by the indophenol.¹⁶⁴

Titration with Methylene-blue Ascorbic acid reduces methylene blue in the presence of light to the leuco compound. This reaction has repeatedly been used^{165 166} and advocated for the quantitative deter-

K. Ott *Angew Chem* 51 537 (1938)

A. Evelyn *J Biol Chem* 115 163 (1936) K. A. Evelyn, H. T. Malloy and C. Rosen *Ibid* 115 (1938)

Guthe and K. K. Nygaard *J Soc Chem Ind* 57 1195 (1938)

L. Mindlin and A. M. Butler *J Biol Chem* 122 673 (1938)

A. Bessey *Ibid* 126 771 (1938)

S. Sebert *Dissertation* Frankfurt a. Main 1931

C. W. Chhoider and A. Okrent *Z physiol Chem* 264 754 (1940)

K. Fujita and T. Ebihara *Biochem Z* 301 779 (1939)

N. Sen Gupta and B. C. Guba *J Ind an Chem Soc* 16 549 (1939)

K. Lund and H. Leck *Nature* 137 784 (1936)

Gál *Ibid* 138 799 (1936)

ever, be overcome by appropriate use of a photoelectric colorimeter, by following the progress of the titration as a function of time and by extrapolation of the results obtained^{143 144 145} The determination of vitamin C in fruits and vegetables may be carried out by any of these methods with a fair degree of accuracy Since the amount of the dehydro form in the living plant is generally insignificant (it is significant, however, in stored materials), approximate values can be obtained by direct titration if the fresh material is extracted sufficiently rapidly with a strong acid, for example, acetic acid,¹⁴⁶ trichloro acetic acid,¹⁴⁷ metaphosphoric acid¹⁴⁸ oxalic acid¹⁴⁹ etc., to diminish the oxidation of vitamin C by the oxidase present in the plant cells and to exclude the titration of glutathione present and if the titration is performed immediately thereafter

Some other modifications have also been proposed Thus, it has been suggested¹⁵⁰ to extract the tissues in the cold with sulfuric acid or with phosphoric acid, to reduce the extract, for example, with hydrogen sulfide, cadmium, zinc aluminum palladium, chromium or titanium at a pH of about 4.5, and to titrate with indophenol before and after an addition of copper sulfate Since the copper ions oxidize preferentially the ascorbic acid, the difference of the two titrations corresponds to the ascorbic acid content

The accuracy of the determination of vitamin C in extracts of animal origin is somewhat limited Liver, for example, may give values up to 20% too high Metaphosphoric acid is particularly useful for the extraction of animal tissues, since it also deproteinizes the extracts¹⁵¹ Foreign reducing materials may be removed with lead acetate¹⁵² or with mercuric acetate¹⁵³ The latter, however, also oxidizes ascorbic acid to a considerable extent to dehydro ascorbic acid

Special methods have been worked out to determine the ascorbic acid concentration in blood plasma¹⁵⁴ and in milk¹⁵⁵ which involve the use of oxalic acid either alone or in mixture with metaphosphoric acid Dehydro ascorbic acid is determined by reduction with hydrogen sulfide

¹⁴³ R. L. Mindlin and A. M. Butler *J. Biol. Chem.* 122 673 (1938)

¹⁴⁴ O. A. Bessey *Ibid.* 126 771 (1938)

¹⁴⁵ W. W. Woessner, C. A. Elvehjem and H. A. Schuette *J. Nutrition* 18 619 (1939)

¹⁴⁶ R. Strohecker and R. Vaubel *Angew. Chem.* 49 666 (1936)

¹⁴⁷ T. W. Burch, L. J. Harris and S. Ray *Biochem. J.* 27 590 (1933)

¹⁴⁸ W. W. Woessner, C. A. Elvehjem and H. A. Schuette *J. Nutrition* 18 619 (1939)

¹⁴⁹ B. Willberg *Z. Unters. Lebensm.* 76 128 (1938)

¹⁵⁰ M. Ott *Angew. Chem.* 54 170 (1941)

¹⁵¹ A. Gupta and T. Ebiyara *Biochem. Z.* 290 172 (1936)

¹⁵² V. A. Deviatkin and V. M. Doroshenko *Ibid.* 280 118 (1935)

¹⁵³ A. Emmerie and M. Eekelen *Biochem. J.* 28 1151 (1934) 30 25 (1936)

¹⁵⁴ R. L. Mindlin and A. M. Butler *J. Biol. Chem.* 122 673 (1938)

¹⁵⁵ W. W. Woessner, C. A. Elvehjem and H. A. Schuette *J. Nutrition* 18 619 (1939)

Vanadium Test¹⁷⁸ Vitamin C yields a blue color, which later changes to green with a reagent prepared from vanadium pentoxide and sulfuric acid

Barac's Azo Test¹⁷⁹ Diazotized sulfanilic acid is reduced by the action of ascorbic acid and forms the orange compound



Sulfanilamide Test¹⁸⁰ A solution of sulfanilamide sodium nitrate, sulfo salicylic acid and urea is mixed with a solution of ascorbic acid, and α dimethyl naphthyl amine is added. The color developed is compared with the color of the same mixture without the vitamin

Selenous Acid Test¹⁸¹ Selenous acid solutions produce an orange red color with ascorbic acid in solution

Gold-trichloride test¹⁸¹ The ability of ascorbic acid to reduce gold trichloride has been used for the determination of vitamin C

Mercuric Chloride Test¹⁸² Mercuric chloride is precipitated from a solution of mercurous chloride by the addition of ascorbic acid

Pitarelli's Test with Cupric Sulfate and Ammonium Thiocyanate¹⁸² A white precipitation occurs upon the addition of cupric sulfate and ammonium thiocyanate and a green color develops upon the addition of further amounts of ammonium thiocyanate to ascorbic acid solutions

Szent-Gyorgyi's Reaction with Ferrous Sulfate A dark violet color develops upon addition of a ferrous sulfate solution to an alkali solution of vitamin C. The color is bleached upon reduction with hyposulfite and can be restored by air oxidation

Test According to Tauber¹⁸³ An acetic acid solution of ascorbic acid yields a blue color when first a solution of ferric cyanide and then a solution of ferric sulfate and phosphoric acid is added

Furfural Test¹⁸⁴ Vitamin C upon boiling with hydrochloric acid forms furfural which is determined with the known aniline phloroglucinol or resorcinol test. Pentoses are included in the values obtained, whereas hexoses and glucuronic acid have a comparatively small furfural producing capacity¹⁸⁵

¹⁷⁸ A. T. Preses *Bol. soc. quim. Pe. u.* 4 22 (1938)

¹⁷⁹ G. Barac *Compt. rend. soc. biol.* 126 61 (1937)

¹⁸⁰ J. V. Scud. and H. D. Ratti *Ind. Eng. Chem. Anal. Ed.* 10 4 0 (1938)

¹⁸¹ A. Emmerle *Acta. B. eva. Veerland. Physiol. Pharmacol. Misc. obsol.* 4 141 (1934)

¹⁸² E. Pittarelli *Biochim. kerap. sper.* 22 100 (1936)

¹⁸³ H. Tauber *M. crochemie.* 17 111 (1935)

¹⁸⁴ J. H. Roe *Science* 80 561 (1934)

¹⁸⁵ G. E. Youngberg *J. Biol. Chem.* 73 599 (1927)

mination of vitamin C The vitamin is extracted from plant or animal material by means of trichloro acetic acid¹⁶⁷ or sulfo salicylic acid¹⁶⁸

Quantitative Determination by Means of Folin's Reagent¹⁶⁹ Vitamin C, extracted from animal or plant material by metaphosphoric acid, has been claimed to be oxidized specifically by mono iodo acetic acid and Folin's reagent,¹⁷⁰ which consists of a solution of molybdenum free sodium tungstate in dilute orthophosphoric acid to which a small amount of bromine water is added A blue color is produced which is measured in a colorimeter This reaction is not very specific and is given, for example, by other dihydroxy compounds and by phenols

A similar color is obtained by the addition of sodium tungstate in sulfuric acid to a solution of the vitamin in metaphosphoric acid followed by the addition of alkali¹⁷¹

Determination by Means of Molybdenum-phosphotungstic acid¹⁷² This reagent produces with vitamin C a violet color which is measured colorimetrically

Test According to Gri Using Ferricyanide and Ammonium Molybdate¹⁷³ Ascorbic acid in trichloro acetic acid solution reduces potassium ferricyanide which upon further addition of ammonium molybdate yields a red brown precipitate Vitamin C solutions from natural sources are purified by the addition of mercuric acetate which precipitates pigments tannins, glutathione cysteine etc

Phosphomolybdic Acid Test^{174, 175} Phosphomolybdic acid produces a blue color in acid solution with ascorbic acid

Bachstetz-Cavallini Reaction with Uranyl-acetate^{176, 177} Uranyl acetate in slightly alkaline solution produces with ascorbic acid a brown color discharged by the alkali followed by precipitation of sodium uranate This reaction serves as a test for differentiating vitamin C from isoascorbic acid, since with the latter compound only a brownish color develops but no precipitation occurs

¹⁶⁷ E Martini and A Bonsignore *Biochem Z* 273 140 (1934) *Boll soc ital biol sper* 9 388 (1934)

¹⁶⁸ W Quensel and K Wachholder *Z physiol Chem* 231 65 (1935) K Wachholder and H H Podesta *Ibid* 239 149 (1936)

¹⁶⁹ A Fujita and T Ebihara *Biochem Z* 290 182 (1936)

¹⁷⁰ O Folin *J Biol Chem* 106 311 (1934)

¹⁷¹ A Fujita D Iwatake and T Miyata *Biochem Z* 277 96 (1935)

¹⁷² N Bezssonoff *Z Vitaminsforsch* 5 193 (1936)

¹⁷³ K V Gri *Microchemie* 23 983 (1938)

¹⁷⁴ H Tillmans and P Hirsch *Z Untersuch Lebensm* 63 2 13 (19 2)

¹⁷⁵ N Bezssonoff *Bil soc chim biol* 16 1107 1133 1160 (1934) H v Euler and D Burström *Biochem Z* 283 153 (1936)

¹⁷⁶ M Bachstetz and G Cavallini *Z physiol Chem* 228 20 (1934)

¹⁷⁷ A Emmenne *Acta Brevis Neerland Physiol Pharmacol Microbiol* 4 141 (1934)

¹⁹⁴ J. Bonner and D. Bonner *Proc Natl Acad Sci U S A* 24 70 (1939) D. Gluck *Z. physiol. Chem.* 245 211 (1937) S. von Hausen *Biochem. Z.* 288, 378 (1936)

A modification of the furfural test is the *osazone furfural method*. This has especially been adapted for the quantitative determination of vitamin C in urine. The vitamin is oxidized to dehydro ascorbic acid by passing through Norite, and separated as a dinitro phenyl osazone. The nitro groups are reduced with stannous chloride followed by hydrolysis of the osazone and the dehydro ascorbic acid obtained is determined by conversion into furfural, etc. Another possibility is to titrate the dinitro phenyl osazone with titanium chloride in acid solution.¹⁸⁵

Cacotheline Test¹⁸⁷ Cacotheline (nitro brucin) produces in hydrochloric acid solution a lilac color with vitamin C and other reducing agents.

Determination of Vitamin C by Its Oxidation to Oxalic Acid Vitamin C is oxidized in acid solution with permanganate. After destroying the excess oxidizing agent with hydrogen peroxide, the oxalic acid produced is determined.¹⁸⁸

Prussian Blue Method An acid ferricyanide solution is easily reduced by vitamin C and converted into Prussian blue by known methods. The amount of blue is measured with a colorimeter.

The *determination of dehydro ascorbic acid in the presence of ascorbic acid can be accomplished* by reaction with 2,4 dinitro phenyl hydrazine. The osazone of the dehydro ascorbic acid precipitates and can be determined gravimetrically or by titration with titanium chloride.

The *rough determination of vitamin C in cells* is carried out by the silver nitrate staining technic of Bourne, Giroud, Leblond and associates.

(c) *Biochemical Methods*

Ascorbic Acid Oxidase Method¹⁸⁹ This method is based on the ability of ascorbic acid oxidase to oxidize, preferentially ascorbic acid. This oxidase, however, is not specific enough to give trustworthy results in the presence of other natural products,¹⁹⁰ especially in the presence of extracts from animal tissues which contain, besides ascorbic acid, other compounds capable of reducing methylene blue.¹⁹¹ It has, furthermore, been observed that a special preparation of oxidase from pumpkin did not react with vitamin C in human urine, spinal fluid and cow's milk.¹⁹

¹⁸⁵ L. Espil and L. Genevois *Bull. soc. chim.* 5 1532 (1938).

¹⁸⁷ L. Rosenthaler *Z. Vitaminsforsch.* 7 126 (1938).

¹⁸⁸ M. Paget and R. Berger *Compt. rend. soc. biol.* 129 960 (1938).

¹⁸⁹ H. Tauber and I. S. Kleiner *J. Biol. Chem.* 110 559 (1935).

¹⁹⁰ G. A. Snow and S. S. Zilva *Biochem. J.* 32 1926 (1938).

¹⁹¹ E. Wachholder and A. Okrent *Z. physiol. Chem.* 264 254 (1940).

¹⁹² N. Bezsonoff and H. Vertruyen *Compt. rend. soc. biol.* 128 407 (1938).

¹¹ J. Bonner and D. Bonner *Proc Natl Acad Sci U S A* 24 70 (1938) D. Glick *Z physiol Chem* 245 211 (1937) S. von Hausen *Biochem Z* 288 378 (1936)

from their seeds and thereby from their natural nutritional resources. Dry seeds do not contain any demonstrable amounts of this vitamin during their inactive rest period, but contain some unknown precursor which is converted into ascorbic acid immediately upon the beginning of germination. Tubers, such as the potato tuber, on the other hand, contain appreciable amounts of vitamin C.

The beneficial effect of ascorbic acid on higher plants can also be demonstrated in other cases. There is a considerable species difference, for example, eggplant shows no response, tobacco plants, however, gain considerably in growth upon administration of ascorbic acid solutions.¹⁹⁷

It is not known where vitamin C is formed in higher plants but it is found regularly in high amounts in all growing parts. Adult parts contain some vitamin C but parts which have turned into wood do not contain any ascorbic acid at all. Haw contains the vitamin in the hull and shell, but not in the seeds.¹⁹⁸ The highest amount is found usually in flowers and in leaves. Petals contain more ascorbic acid than pistils, stamens and calyces.¹⁹⁹

Light seems to have a beneficial effect upon the production of ascorbic acid in plants.²⁰⁰ This finding might probably be linked with the observation that red and violet flowers generally contain more active material than white and yellow flowers.²⁰¹ The concentration of ascorbic acid in plant leaves is also a function of the light received and fluctuates considerably during the day, the maximum being reached, for example, in potato leaves in the forenoon.²⁰²

The mechanism of the ascorbic acid action in plants is largely unknown. The conception of a participation in the oxidation reduction systems of the living plant will be discussed with the corresponding action in animal tissues. (See page 326.)

The physiology of vitamin C in microorganisms differs considerably with the species. Thus some bacteria need vitamin C and are apparently able to synthesize it.²⁰³ This question has especially been studied on lactic acid bacteria and it has been found that some strains show a definite growth response to vitamin C added to the culture medium,²⁰⁴ while others are re-

¹⁹⁷ R. Dennison *Science* 92 17 (1940)

¹⁹⁸ H. Winckelmann *Hippokrates* 9 714 (1938)

¹⁹⁹ H. Mituda *J. Agr. Chem. Soc. Japan* 14 1228 (1938)

²⁰⁰ M. E. Reid *Am. J. Botany* 25 702 (1938)

²⁰¹ H. Mituda *J. Agr. Chem. Soc. Japan* 14 1228 (1938)

²⁰² A. M. Smith and J. Gillies *Biochem. J.* 34 1312 (1940)

²⁰³ G. Bourne and R. Allen *Australian J. Exptl. Biol. Med. Sci.* 13 165 (1935)

²⁰⁴ O. Rahn and C. P. Hegarty *Proc. Soc. Exptl. Biol. Med.* 38 218 (1938). O. Rahn, C. P. Hegarty and R. E. Deuel *J. Bact.* 35 647 (1938). A. Sartory, R. Sartory and J. Meyer *Compt. rend.* 206 1414 (1938). J. G. Davis and J. McClellmont *J. Dairy Research* 10 94 (1939)

tarded and some apparently do not respond either way Vitamin C is synthesized for example, by *Bacillus prodigiosus*²⁰⁸ as proved by chemical and biological determination It has also been demonstrated that some protozoa need an external supply of ascorbic acid for optimal growth²⁰⁸

14 Animal Physiology

Of all the vitamins vitamin C has received the widest attention in a large number of various investigations relating to different phases of physiological interest Nevertheless the knowledge about the general physiology, the metabolism and the mechanism of the vitamin C action is still fragmentary and the fundamentals of these subjects are still unknown

(a) Metabolism

Ascorbic acid is absorbed by the tissues of the intestinal tract²⁰⁷ principally of the small intestine After oral ingestion of vitamin C the vitamin level in blood plasma rises to a maximum within about 1.5 hours but occasionally, for example after an intake of strawberries or cauliflower a considerably longer period of time elapses before the vitamin level in the blood is increased²⁰⁸ In any event the increase is only temporary The vitamin is transported with the blood throughout the entire organism and excess amounts are secreted in the urine²⁰⁹ where it appears mostly in the free form, but to a small extent also in a combined form²¹⁰ Ascorbic acid given intravenously or subcutaneously raises temporarily the vitamin C content of the blood, but is excreted within one to three hours^{211 212} More prolonged effects are obtained if the vitamin is injected intramuscularly Thus, however, causes sloughing due to the acidity of the vitamin The preferred method in cases of necessary parenteral administration is to inject salts for example, sodium salt or salts of organic amines for example of mono ethanol amine²¹³

²⁰⁸ K. H. Busing and F. Peters *Biochem. Z.* 304: 134 (1940)

²⁰⁹ M. Lwoff *Compt. rend.* 206: 540 (1938) *Compt. rend. soc. biol.* 130: 406 (1939) R. Caillaud *Compt. rend. soc. biol.* 127: 861 (1938) 131: 964 (1939) 138: 319 (1939)

²¹⁰ S. S. Zilva *Biochem. J.* 29: 100 (1935)

²¹¹ E. N. Todhunter *Am. Inst. Nutrition Abstracts J. Nutrition* 21: 12 (1941)

²¹² L. J. Harris and S. N. Ray *Lancet* 1: 71 (1935) H. v. Euler and M. Malmberg *Biochem. Z.* 279: 338 (1935)

²¹³ B. C. Guha and P. N. Sen Gupta *Nature* 141: 874 (1938) H. Scarborough and C. P. Stewart *Biochem. J.* 31: 232 (1937) *Nature* 142: 40 (1938)

²¹⁴ F. E. Hawley and D. J. Stephens *Proc. Soc. Exptl. Biol. Med.* 34: 84 (1937) M. van Eekelen and M. Heinemann *J. Clin. Invest.* 17: 293 (1938)

²¹⁵ E. P. Ralli, G. J. Fredman and M. Kaslow *Proc. Soc. Exptl. Biol. Med.* 36: 52 (1937) I. S. Wright, A. Lilenfeld and E. MacLennan *Arch. Internat. Med.* 60: 264 (1937) J. M. Faulkner and P. H. L. Taylor *J. Clin. Investigation* 17: 69 (1938)

²¹⁶ E. L. Lozner, F. J. Poble and P. H. L. Taylor *New England J. Med.* 220: 987 (1939)

Tissues and body fluids contain various amounts of this vitamin. Normal human blood plasma contains about 1.2 mg ascorbic acid in 100 cc.²¹⁴ There are no real storage organs for this vitamin, although some organs contain increased amounts. Among these the adrenal gland contains the most. It has been shown, however, that, for example, in guinea pigs the amount of ascorbic acid in this gland cannot be increased, even by feeding excess amounts of vitamin C.²¹⁵ Generally, tissues of high metabolic activity have the highest vitamin C content. Thus, young tissues contain more vitamin C than older tissues, as has been shown on the thymus²¹⁶ and the corpus luteum.²¹⁷ Elderly people generally have less vitamin C reserves than young people. Vitamin C is secreted in the milk. Cow's milk contains on the average about 22 mg/l. Human milk contains several times more vitamin C than cow's milk (about 75 mg/l), since babies, but not calves, need an external supply of this vitamin. Colostrum contains somewhat more vitamin C.²¹⁸ The ascorbic acid secretion in cow's milk varies somewhat with the season. The highest amount has been found in the late summer or early fall.²¹⁹ Ascorbic acid is also at times excreted in the sweat.²²⁰ The main excretion is through the urine, as stated before. The excretion occurs within four to six hours following ingestion.²²¹ Some ascorbic acid is also excreted in the feces.²²

(b) Physiological Action

The most obvious property of ascorbic acid is the reversible oxidation and reduction capacity, and much speculation and experimental work arose from the idea of correlating this behavior with the mechanism of the vitamin action. It seems established that under physiological conditions the reversibility of the reducing capacity of ascorbic acid exists, although this reversibility is only partial in *in vitro* experiments. The instability of the oxidized form, dehydro ascorbic acid, even in the intact cell, is probably the reason for the fact that the organism needs a relatively greater amount of this vitamin than of the other vitamins.

²¹⁴ A. F. Abt, C. J. Farmer and I. M. Epstein, *J. Pediatrics* 8, 1 (1936). D. J. Stephens and E. E. Hawley, *J. Biol. Chem.* 115, 653 (1936).

²¹⁵ G. Mounquand, M. Dauvergne and V. Edel, *Compt. rend.* 209, 1023 (1939).

²¹⁶ D. Glick and G. R. Biskind, *J. Biol. Chem.* 113, 27 (1936); 114, 1 (1936).

²¹⁷ B. C. Guha and P. N. Sen Gupta, *Nature* 141, 974 (1938).

²¹⁸ F. Schlemmer, B. Bleyer and H. Cahnmann, *Biochem. Z.* 254, 187 (1932).

²¹⁹ A. D. Holmes, F. Tripp, E. A. Woelffer and G. H. Satterfield, *J. Nutrition* 17, 187 (1937).

²²⁰ A. Lihensfeld, I. S. Wright and E. MacLenathen, *Proc. Soc. Exptl. Biol. Med.* 35, 184 (1936). R. E. Bernstein, *Nature* 140, 684 (1937).

²²¹ E. E. Hawley and D. J. Stephens, *Proc. Soc. Exptl. Biol. Med.* 34, 854 (1936). M. van Eekelen and M. Heinemann, *J. Clin. Investigation* 17, 293 (1938).

²² H. Chinn and C. J. Farmer, *Proc. Soc. Exptl. Biol. Med.* 41, 561 (1939).

It has been postulated that there exist special enzyme systems which take care of both the oxidation and the reduction of ascorbic acid. Thus an enzyme has been found in blood²²³ and in plant juices²²⁴ which reduces dehydro ascorbic acid'. On the other hand glutathione has been shown to be the most effective reductant and protective agent for vitamin C in the living animal and in some plant cells²²⁵.⁶ Besides glutathione other compounds with fixed sulphhydryl groups exert reducing capacities upon the oxidized form of vitamin C. Also certain purines such as xanthine, uric acid and theophylline but not caffeine and theobromine, and creatinine but not creatine have been shown experimentally to protect the vitamin against oxidation²²⁷. The opposite reaction, the oxidation of ascorbic acid is much easier to demonstrate and is carried out by a special enzyme ascorbic acid oxidase. The existence of this enzyme in plants is established,²²⁸²²⁹²³⁰ but its occurrence in animal tissues is questionable. Ascorbic acid oxidase has the constitution of a copper protein²³⁰ and is specific for the stereochemical configuration of L ascorbic acid.³¹ Also, various polyphenylases are able to oxidize ascorbic acid in plants²³². The search for the nature of the ascorbic acid oxidizing enzyme in animal tissues has brought forward many experimental evidences which partly support and partly contradict the conception of the constitution of the enzyme as a copper protein. It seems plausible to assume that a number of different oxidation reduction systems act on ascorbic acid in the living organism. Thus conclusive evidence has been presented that ascorbic acid is rapidly oxidized by cytochrome oxidase plus cytochrome c²³³. The question of the nature of the mechanism of the ascorbic acid action should therefore be separated from any discussion of the reversible oxidation reduction action of this vitamin until specific reactions of physiological importance in various organs, cells, body fluids etc., have been established.

A number of observations along these lines have been made but it is impossible at the moment to correlate these findings and to decide which ones

²²³ J. H. Roe and G. L. Barnum *J. Nutrition* 11: 359 (1936)

²²⁴ E. M. Crook and F. G. Hopkins *Biochem. J.* 32: 1356 (1938)

²²⁵ H. Borsook, H. W. Davenport, C. E. P. Jeffreys and R. C. Warner *J. Biol. Chem.* 117: 237 (1937)

²²⁶ F. G. Hopkins and E. J. Morgan *Biochem. J.* 30: 1446 (1936)

²²⁷ K. V. Giri and P. V. Krishnamurthy *Nature* 147: 59 (1941)

²²⁸ A. Szent-Györgyi *J. Biol. Chem.* 90: 385 (1931)

²²⁹ Z. I. Kertész, R. B. Dearborn and G. L. Mack *ibid.* 116: 717 (1936)

²³⁰ P. L. Lovett-Jones and J. M. Nelson *J. Am. Chem. Soc.* 62: 1409 (1940)

²³¹ H. Rosenberg *Skand. Arch. Physiol.* 76: 119 (1937)

²³² D. Keilin and T. Mann *Proc. Roy. Soc. (London)* B125: 187 (1938) F. Kubowitz *Biochem. Z.* 299: 3 (1938)

²³³ D. Keilin and E. F. Hartree *Proc. Roy. Soc. (London)* B125: 171 (1938) E. Stott, C. J. Hartree, M. O. Schultze and C. G. King *J. Biol. Chem.* 122: 407 (1938)

of all fibrous tissues and non epithelial cement substances²⁵⁴⁻⁵⁵ During avitaminosis, these fibrils or collagen are not formed. These phenomena are in close relationship to the disturbance of the calcium metabolism during times of vitamin C depletion, which affects growth and maintenance of bones and teeth and which sometimes appears similar to that observed during vitamin D deficiency. Also the phenomena of hemorrhagic lesions associated with vitamin C depletion appear to be connected with this basic function of the ascorbic acid. Experimental studies have also shown the particular presence of vitamin C in Golgi cells in the cerebral cortex and the cord, the function of which cells is to bring neighboring cells into relation to each other, and in the mitochondria, which form an essential part of the cytoplasm.²⁵⁶

Of particular interest is the reaction of the human organism toward ascorbic acid administration in cases of various poisonings. It has already been pointed out in the section on the Biogenesis of vitamin C (page 312) that the organism of ascorbic acid producing animals reacts to the administration of chemicals, especially of ketones, by increasing the ascorbic acid excretion. In human beings beneficial effects of large doses of vitamin C have been recorded in cases in which the vitamin may act as a detoxicant. Cases have become known in which toxic doses of drugs, for example, tyrosine,²⁵⁷ chemicals²⁵⁸ for example lead²⁵⁹ and arsenic²⁶⁰ compounds, benzene,²⁶¹ etc., toxins²⁶² virus and substances producing anaphylaxis,²⁶³ etc., have had no toxic effect when administered simultaneously with vitamin C. It seems that ascorbic acid combines with some of these substances and is excreted in such combination. Similar detoxification effects are observed toward diphtheria toxins²⁶⁴⁻²⁶⁵ tuberculosis,²⁶⁶ and

²⁵⁴ S. B. Wohlbach and P. R. Howe *Arch Path* 11 (1926) S. B. Wohlbach *Am J Path* 9 689 (1933) *J Am Med Assoc* 108 7 (1937)

²⁵⁵ A. v. Jency and E. Toró *Arch path Anat (Virchow's)* 298 87 (1936)

²⁵⁶ A. Giroud and C. P. Leblond *Anat Record* 68 113 (1937) G. Bourne and R. Allen *Australian J Exptl Biol Med Sci* 13 165 (1935)

²⁵⁷ R. R. Sealock and H. F. Silberstein *Science* 90 517 (1939)

²⁵⁸ M. Vauthey *Ann maladies vénériennes* 32 98 (1937) *Ann dermatol syphilol* 8 568 (1937)

²⁵⁹ H. N. Holmes, E. J. Amberg and K. Campbell *Science* 89 372 (1939) *J Lab Clin Med* 24 1119 (1939)

²⁶⁰ M. B. Sulzberger and B. L. Oser *Proc Soc Exptl Biol Med* 32 716 (1935) F. E. Corrao *Can Med Assoc J* 36 392 (1937) S. Landfisch *Polaka gas lek* 16 575 (1937) I. Dainow *Presse méd* 45 1670 (1937)

²⁶¹ A. Meyer *Z Vstamnforsch* 6 83 (1937)

²⁶² C. W. Jungeblut *J Immunol* 33 203 (1937)

²⁶³ F. Diehl *Klin Wochschr* 18 956 (1939)

²⁶⁴ A. Sigal and C. G. King *J Pharmacol Exptl Therapy* 59 468 (1937) 61 1 (1937)

²⁶⁵ C. G. King and M. L. Menten *J Nutrition* 10 129 141 (1935) C. K. Greenwald and E. Harde *Proc Soc Exptl Biol Med* 32 1157 (1935)

²⁶⁶ W. W. Jetter and T. S. Bumbalo *Am J Med Sci* 195 362 (1938) M. N. Rudra *Current Sci* 8 210 (1939)

many other infectious diseases²⁶⁷ During such times the amount of ascorbigen excreted in the urine is increased²⁶⁸ Guinea pigs which died from diphtheria intoxications showed reduced vitamin C content of the suprarenals²⁶⁹ whereas no significant differences were found between the vitamin C content of the suprarenals of animals injected with a sublethal dose of diphtheria toxins and those of normal animals²⁷⁰ Bacterial toxins can cause a decrease of as much as 50 to 85% of the normal vitamin C content of the adrenals²⁷¹

In this connection the correlation of vitamin C to the complement is of importance The complement is a thermolabile protein substance in the blood serum which destroys bacteria and other cells The complement exhibits a reversible oxidation reduction potential the maintenance of which is to a great extent a function of the ascorbic acid content of the blood serum²⁷²

Vitamin C has also been linked with the amino acid metabolism It has, for example been shown *in vitro* that dehydro ascorbic acid dehydrates amino acids for example, leucine, with the formation of ammonia and strongly reducing acidic compounds, probably keto acids²⁷³

A close relationship of vitamin C to the carbohydrate metabolism has for example, been demonstrated in the case of guinea pigs in which the capacity for metabolizing glucose²⁷⁴ or dextrose²⁷⁵ is moderately lowered in the prescorbutic and scorbutic stage of vitamin C deficiency Vitamin C increases blood sugar in cases of hypoglycemia and in prolonged usage tends to prevent hypoglycemia In schizophrenics given insulin shock treatment, the vitamin raises the blood sugar and enables the patient to be revived more quickly than by sugar administration Vitamin C is thus a factor in carbohydrate metabolism This has also been shown in a disease in which there is a disturbance of muscle glycogen metabolism²⁷⁶

²⁶⁷ D Perla and J Marmorston *Arch Path* 23 683 (1937)

²⁶⁸ B Ghosh *J Ind an Chem Soc* 16 241 (1939)

²⁶⁹ E Harde *Compt rend* 199 618 (1934) C W Jungeblut and R. L Zwemer *Proc Soc Exptl. Biol Med* 32 1229 (1934-35)

²⁷⁰ C C Torrance *J Biol Chem* 132 575 (1940)

²⁷¹ C M Lyman and C G King *J Pharmacol Exptl Therapy* 56 209 (1936) L J Harris R Passmore and W Pagel *Lancet* 2 183 (1937) C C Torrance *J Bact* 33 645 (1937)

²⁷² E E Ecker L Pillemer D Wertheimer and H Grady *J Immunol* 34 19 (1938) E E Ecker L Pillemer J J Griffiths and W F Schwartz *J Am Med Assoc* 112 1449 (1939)

²⁷³ H v Euler P Karrer and F Zehnder *Helv Ch m Acta* 17 157 (1934)

²⁷⁴ A Sigal and C. G King *J Biol Chem* 116 489 (1936)

²⁷⁵ A Sigal and C. G King *J Pharmacol Exptl Therapy* 39 468 (1937) 61 1 (1937)

²⁷⁶ E Wille *Deut med Wochschr* 65 1117 (1937)

It is also suggested²⁷⁷ that vitamin C, copper and protective substances, like glutathione, which occur together in all tissues, play an important role in the regulation of the activity of tissue phosphatases

(c) *Relation of Vitamin C to Other Vitamins, Hormones, Etc*

As stated previously in the general chapter on vitamins, there is no synergistic or antagonistic action of one vitamin to another²⁷⁸ Vitamin C has the special faculty of detoxifying a large variety of different compounds including toxic doses of other vitamins In animals which have the power of synthesizing their own supply of ascorbic acid the observation has generally been made that the amount produced is reduced at times of low vitality Therefore in rats fed a vitamin A free diet the ascorbic acid content of the heart and probably also of the kidney and the thymus is significantly reduced²⁷⁹ Similar reductions in the ascorbic acid concentration in various tissues and endocrines have been observed in vitamin B₁ and riboflavin deficiencies, but no noteworthy changes occurred as a result of B₁ avitaminosis²⁷⁹

A close relationship of vitamin C to various hormones is noted since during avitaminosis a decreased hormone secretion is observed from those glands which normally contain high concentrations of vitamin C, for example, pituitary, pancreas, adrenal, thyroid, liver, intestinal wall²⁸⁰ In particular, it has been observed that vitamin C is necessary for the utilization of the adrenal cortex hormones, especially for the salt metabolism controlled by these hormones²⁸¹ There seems to exist an antagonism between vitamin C and thyroxine Administration of this hormone reduces the vitamin C content of liver, adrenals, etc This effect can probably be linked with the property of ascorbic acid of detoxifying harmful compounds

The relationships of vitamin C to various enzymes and to traces of metals, especially to manganese and copper, have already been discussed

15 Avitaminosis and Hypovitaminosis

The state of hypovitaminosis which is quite common among human beings is characterized by impairment of physiological functions Guinea

²⁷⁷ K. V. Giri *Biochem J* 33 309 (1939)

²⁷⁸ P. E. Simola *Acta Soc Med Fenn Duodecim* 16 No 3 1 (1933) *Ber ges Physiol experl Phar* makol 83 312 A. Scheunert *Naturwissenschaften* 28 297 (1940)

²⁷⁹ B. Sure, R. M. Theis and R. T. H. Erelson *J Biol Chem* 129 245 (1939)

²⁸⁰ A. Sigal and C. G. King *Ibid* 116 489 (1936)

²⁸¹ J. L. Svirbely *Ibid* 116 543 (1936)

pigs during hypovitaminosis are very sensitive to injury from diphtheria and other toxins²⁸² and toward infectious diseases²⁸⁴. In humans, a depletion of the vitamin C reserves occurs during many diseases, for example during fever,²⁸⁵ tuberculosis²⁸⁶ etc. During avitaminosis the vitamin C level in blood serum and in the urine is lowered and the normal metabolic activity is decreased. More severe deficiency causes sore and swollen joints, edema, shortness of breath and a decline in weight. The clinical symptoms of vitamin C avitaminosis are summarized under the term scurvy and are mainly characterized by hemorrhagic conditions. The actual place of these is largely influenced by growth and stress²⁸⁷. Thus in the growing human hematomas, steoporosis, bone pains etc., occur. The site of hemorrhages in adults is determined mainly by physical stress. During avitaminosis bones cease to grow and the normal junctions are replaced by connective tissue which contains calcified cartilages, but is devoid of osteoid tissue. This phenomenon can be seen in roentgenograms. The enamel cementum and most predominantly, the dentine change in structure, become resorbed and porotic and the newly formed material is of inferior strength (osteodentine). The gingiva, the gum of the jaws and surrounding the teeth swells up, becomes spongy and bleeds easily. In severe cases hemorrhagic lesions are also observed in muscles, eyes (cataract²⁸⁸) and skin (lesions of the acne type²⁸⁹). Furthermore, a typical anemia of scurvy develops. Concomitant signs of a vitamin C depletion are atrophy of the glands of internal secretion and of the lymphatic tissues. The clinical symptoms of vitamin C deficiency in infants are known under the name Moller-Barlow's disease.

Vitamin C shortage in the organism is most dangerous in cases of bone fractures²⁹⁰ and wound healing²⁹¹ which are not cured rapidly and properly unless enough vitamin C is available. Cases have also been reported²⁹² showing that subnormal dark adaptation of the eye may be caused by vita-

²⁸² A. Sigal and C. G. King, *J. Pharmacol. Exptl. Therapy* 59: 468 (1937); 61: 1 (1937).

²⁸³ C. G. King and M. L. Menten, *J. Nutrition* 10: 129-141 (1935). C. K. Greenwald and E. Harde, *Proc. Soc. Exptl. Biol. Med.* 32: 1157 (1935).

²⁸⁴ D. Ferla and J. Marmorston, *Arch. Path.* 23: 683 (1937).

²⁸⁵ K. Daum, K. Boyd and W. D. Paul, *Proc. Soc. Exptl. Biol. Med.* 40: 179 (1939). Falke, *Klin. Wochschr.* 18: 818 (1933).

²⁸⁶ W. W. Jitter and T. S. Bumb, *Am. J. Med. Sci.* 195: 369 (1938). M. N. Rudra, *Current Sci.* 8: 210 (1939).

²⁸⁷ G. D. Ildorf, *J. Exptl. Med.* 50: 293 (1939).

²⁸⁸ H. Scarborough and C. P. Stewart, *Biochem. J.* 31: 2232 (1937).

²⁸⁹ M. Lorenza, *Minerva Medica* 30: 735 (1939).

²⁹⁰ H. H. Hake, *Deutscher Z. Chir.* 245: 530 (1935).

²⁹¹ W. Aron, *Die Nahrungsmittel des Kindes*, Berlin 1928. T. H. Lanman and T. H. Ingalls, *Ann. S. G.* 105: 616 (1937). M. Taffel and S. C. Harvey, *Proc. Soc. Exptl. Biol. Med.* 38: 418 (1938). A. W. Allen, *Intern. Abstracts* 5: 69: 111 (1939).

²⁹² M. S. Kimble and E. S. Gordon, *J. Biol. Chem.* 128: LII (1939).

min C depletion at times when enough vitamin A and riboflavin were ingested. However a possible relationship of vitamin C to certain forms of cataract has been denied.²⁹³

Administration of vitamin C in human therapy is also indicated in cases of excessive bleedings (with the exception of hemophilia),²⁹⁴ rheumatic fever²⁹⁵ and arthritis, anaphylaxis, drug hypersensitivity, certain forms of allergy, lead and arsenic poisoning,²⁹⁶ and Addison's disease in order to decrease the degree of pigmentation of the skin.²⁹⁷ In cases of disturbances of the gastrointestinal tract and especially in gastric and duodenal ulcers,²⁹⁸ vitamin C deficiencies occur quite often due to intertestinal destruction of the vitamin or to poor absorption. Thus chronic scurvy is often diagnosed as pyorrhea.²⁹⁹ In such cases the vitamin is administered parenterally, preferably by intramuscular injection.³⁰⁰ Since vitamin C is also a factor in carbohydrate metabolism, an administration of this vitamin has been suggested in those illnesses in which there is a disturbance of the glyco-gen metabolism, in hypoglycemia, etc.³⁰¹

In guinea pigs, typical changes of the female sex organs occur. The development of the follicles becomes greatly retarded and no corpora lutea develop at all. If the state of avitaminosis endures over a prolonged length of time, these changes cannot be repaired by the administration of vitamin C.

(a) Clinical Test Methods

Clinically the detection of a vitamin C deficiency is of importance especially when a state of hypovitaminosis is suspected. The following methods are now generally used.

Blood Test The ascorbic acid content of normal human blood plasma is about 1.2 mg per 100 cc, but this value decreases considerably upon a

²⁹³ J. Urbanek *Klin Monatsbl f Augenh* 101 671 (1938)

²⁹⁴ E. Vogt *Munch med Wochschr* 82 263 (1935) H. O. Neumann *Klin Wochschr* 15 368 (1936) D. K. Miller and C. P. Rhoades *J Clin Investigation* 15 462 (1936)

²⁹⁵ J. F. Rinehart *J Lab Clin Med* 21 597 (1936) J. F. Rinehart L. D. Greenberg M. B. Olney and F. Choy *Arch Internal Med* 61 552 (1938) M. A. Abbasy N. G. Hill and L. J. Harris *Lancet* 2 1413 (1936) M. A. Abbasy L. J. Harris and P. Elliman *Ibid* 2 181 (1937)

²⁹⁶ H. N. Holmes E. J. Amberg and K. Campbell *Science* 89 322 (1939) *J Lab Clin Med* 24 1119 (1939)

²⁹⁷ T. Cornbleet *Arch Dermatol Syphilol* 35 471 (1937) J. F. Wilkinson and C. A. Ashford *Lancet* 2 967 (1936) A. F. Abt and C. J. Farmer *J Am Med Assoc* 111 1555 (1938)

²⁹⁸ A. B. Rivers and L. A. Carlson *Proc Staff Meetings Mayo Clinic* 12 383 (1937) G. Bourne *Brit Med J* 1 560 (1938) B. Portnoy and J. F. Wilkinson *Ibid* 1 554 (1938)

²⁹⁹ D. Weisberger *J Conn State Med Soc* 1 492 (1937)

³⁰⁰ M. Vauthey *Rev Gastroenterol* 6 337 (1939)

³⁰¹ E. Wille *Deut med Wochschr* 63 1117 (1937)

vitamin C deficient diet. In the prescorbutic state the content is about 8 mg and by the time clinical symptoms of scurvy become evident the vitamin C content of blood plasma is around 0.5 mg.^{302 303} When vitamin C is administered in excess amounts the blood level may approach a value of 2.00 mg per 100 cc.

The actual determination of vitamin C in blood is carried out, for example, by titration with the indophenol indicator.³⁰⁴ It is necessary to deproteinize the blood by means of a strong acid, for example trichloroacetic acid, tungstic acid³⁰⁵ or metaphosphoric acid,³⁰⁶ and thereafter to separate the clear blood plasma. This method has also been adapted for micro-work.³⁰⁷ The Prussian blue method,³⁰⁸ the methylene blue³⁰⁹ and some of the other methods described under "chemical methods of determining vitamin C" (page 316) have occasionally been used. Since the precipitation of protein material sometimes carries ascorbic acid along, it has also been suggested to titrate blood serum directly with indophenol in the presence of hydrochloric acid.³¹⁰ It is necessary to determine the vitamin C content of blood serum immediately after the separation of the red blood cells since the vitamin C content decreases rapidly.³¹¹ On the other hand, these determinations of vitamin C in the blood plasma are of fair accuracy if carried out properly, since no dehydroascorbic acid is present in fresh blood, but is found only as an artefact.

Urine Test. The daily urinary output of vitamin C in avitaminotic humans is greatly diminished in comparison to the normal output. Clinical determinations are made on the urine of patients before and after an oral or preferably after an intramuscular administration of moderate doses of vitamin C. Since a normal human being needs about 25–50 mg of ascorbic acid daily, the response of the urinary excretion to the administration of this amount is determined.^{312 313} The urinary output of ascorbic acid of

³⁰² J. M. Faulkner and P. H. L. Taylor *J. Clin. Investigation* 17: 69 (1938). I. S. Wright *Ann. Internal Med.* 12: 516 (1938). H. Lund *Klin. Wochschr.* 16: 1035 (1937). G. A. Goldsmith and G. F. Hinger *Arch. Internal Med.* 63: 531 (1939). F. N. Todhunter and R. C. Robbins *J. Nutrition* 19: 33 (1940).

³⁰³ C. J. Farmer and I. M. Epstein *J. Pediatrics* 8: 1 (1936).

³⁰⁴ R. L. Mindlin and A. M. Butler *J. Biol. Chem.* 122: 673 (1938).

³⁰⁵ P. H. L. Taylor, D. Chase and J. M. Faulkner *Biochem. J.* 30: 1119 (1936).

³⁰⁶ C. J. Farmer and A. F. Abt *J. Pediatrics* 8: 1 (1936). *Proc. Soc. Exptl. Biol. Med.* 38: 399 (1938).

³⁰⁷ C. J. Farmer and A. F. Abt *Proc. Soc. Exptl. Biol. Med.* 34: 146 (1936).

³⁰⁸ H. Tauber and I. S. Kleiner *J. Biol. Chem.* 110: 559 (1935).

³⁰⁹ H. Lund and H. Lieck *Klin. Wochschr.* 16: 555 (1937). H. Wahren *Ibid.* 16: 1496 (1937). A. Imby and T. Wirth *Ibid.* 16: 746 (1937).

³¹⁰ N. Berend and M. Fischer *Biochem. Z.* 291: 271 (1937).

³¹¹ R. J. Kassar and J. H. Roe *J. Biol. Chem.* 133: 579 (1940).

³¹² W. B. Belser, H. M. Hauck and C. A. Storvick *J. Nutrition* 17: 513 (1939).

³¹³ L. J. Harris and S. N. Ray *Lancet* 1: 71 (1935). H. E. Archer and G. Graham *Ibid.* 1: 710 (1936). Sloan *J. Lab. Clin. Med.* 23: 1015 (1938).

healthy people varies considerably and can be influenced by a change in the acid base balance of the food consumed ³¹⁴

The exact actual determination of the total urinary excretion of vitamin C, comprising ascorbic acid, dehydro ascorbic acid and ascorbic acid bound to protein material, is rather difficult. It has, for example, been suggested to use the titration with indophenol in various modifications³¹⁵ or with methylene blue,³¹⁶ the colorimetric determination with molybdo phosphotungstic acid³¹⁷ or to use the osazone furfural method ^{318 319}

A certain small amount of ascorbic acid is said to be excreted in combined form ³²⁰. This amount is not included in all the results of ascorbic acid determination, unless this part of the total ascorbic acid content is liberated from the combined form. During many diseases, particularly during times of fever, diphtheria, etc., the amount of the combined form is considerably increased ³²¹

Skin Capillary Fragility Test One of the first signs of a state of clinical scurvy (and of a vitamin P deficiency) is the considerably lowered capillary resistance ^{3 322}. A relative quantitative picture of the vitamin C depletion of a patient can be obtained by measuring the fragility. This is done, for example, in the compression test, by pinching the skin with the finger for one minute and investigating the number and severity of the petechiae (hemorrhagic spots) produced. A more exact effect is obtained by applying pressure with a sphygmomanometer³²⁴ which is inflated to a pressure below the diastolic pressure of the pulse. A suction method can also be used, applying negative pressure. The average resistance of human skin is about 30 cm Hg but varies considerably with different parts of the skin.

Roentgenographic Examination of Bones ³²⁵ Since a slight hypovitaminosis causes no changes in the bone structure only more severe cases can be detected by roentgenographic studies. On the other hand, scurvy may be manifest in the skeleton without other clinical symptoms. The patho-

³¹⁴ E. E. Hawley, J. P. Frazer, L. L. Button and D. L. Stevens, *J. Nutrition* 12, 215 (1936).

³¹⁵ A. Jezler and W. Niederberger, *Klin. Wochschr.* 15, 710 (1936). J. Gander and W. Niederberger, *Munch. Med. Wochschr.* 83, 1386, 2074 (1936). H. Kaiser, *Süddent. Apoth. Ztg.* 1936, No. 85.

³¹⁶ H. Lund, *Klin. Wochschr.* 16, 1085 (1937).

³¹⁷ N. Bezsonoff and E. Stoerr, *Z. Vitaminforsch.* 5, 193 (1936).

³¹⁸ J. H. Roe and J. M. Hall, *J. Biol. Chem.* 128, 329 (1939).

³¹⁹ L. Espal and L. Genevois, *Bull. soc. chim.* 5, 1532 (1938).

³²⁰ S. Banerjee, *J. Indian Chem. Soc.* 17, 463 (1940).

³²¹ B. Ghosh, *Ibid.* 16, 241 (1939).

³²² G. Dalldorf, *J. Exptl. Med.* 58, 289 (1931). *Am. J. Diseases Children* 46, 794 (1933).

³²³ G. F. Göthlin, *Skand. Arch. Physiol.* 61, 295 (1931).

³²⁴ I. S. Wright and A. Lal, *enfeld Arch. Internal Med.* 57, 241 (1936).

³²⁵ E. A. Park, H. G. Guild, D. Jackson and M. Bord, *Arch. Disease Childhood* 10, 265 (1935).

logical symptoms are encountered in the peripheral region and the end of the shaft of the long bones

Adrenal Cortex Examination For autopsy purposes a rough determination of the vitamin C content is made by soaking the opened gland in a silver nitrate solution. In cases of deaths caused by vitamin C depletion very little silver precipitation occurs

The Intradermal Test This method is based on the observation that a solution of dichlorophenol indophenol injected under the epithelium discolorizes.³¹⁶ It has been estimated that a discoloration time of a given amount of the dye of five minutes indicates saturation with vitamin C and ten minutes or more indicates hypovitaminosis.³²⁷ This test however, proved to lack sufficient specificity for clinical work.³²⁸

16 Hypervitaminosis

A state of vitamin C hypervitaminosis is unknown. It has been impossible to produce any toxic symptoms with guinea pigs by feeding excess amounts of this vitamin and no increase in the vitamin C content of the organs over their normal levels could be detected.³²⁹ No toxic signs were observed in human beings who were given doses of from 1 to 6 g orally or intravenously.³³⁰ Occasional vagotonic symptoms are attributed to idiosyncrasy or drug sensitivity.³³¹ Ingestion of ascorbic acid has a slight diuretic effect,³³² less than that caused by theobromine, but greater than the diuresis induced by digitalis.³³³ In animals the blood pressure rises somewhat upon injections of ascorbic acid.³³⁴

17 Requirements³³⁵

Of the entire living world only man, the other primates, the guinea pig and a few microorganisms (see under Physiology, page 324) are known to require an external supply of vitamin C. All other animals and plants also need vitamin C but are able to synthesize it; that is, ascorbic acid is a hormone for all these organisms. Guinea pigs need from 1 to 2 mg of

³¹⁶ H. Rotter, *Nat.* 139: 717 (1937)

³¹⁷ B. Portnoy and J. F. Wilkinson, *Lancet* 1: 38 (1938)

³¹⁸ H. G. Poucher and C. H. Stubenrauch, *J. Am. Med. Assoc.* 111: 303 (1938)

³¹⁹ G. Mouriquand, M. Duvigneau and V. Edel, *Compt. rend.* 209: 1073 (1939)

³²⁰ A. F. Abt and C. J. Farmer, *J. Am. Med. Assoc.* 111: 1533 (1938)

³²¹ F. Widenbauer, *Klin. Wochschr.* 15: 1155 (1936)

³²² M. A. Abbasy, *Biochem. J.* 31: 339 (1937)

³²³ W. Evans, *Lancet* 1: 308 (1938)

³²⁴ M. Kasahara and R. Kawamura, *Klin. Wochschr.* 16: 1543 (1937)

³²⁵ S. L. Smith, *J. Am. Med. Assoc.* 111: 1753 (1938)

ascorbic acid daily The average optimal intake for human beings is about 50-100 mg The fact that infants require vitamin C should be especially emphasized, since cow's milk does not contain sufficient amounts and human milk is given usually only over a relatively short period of time and its vitamin C content decreases after a few weeks Infants need 3 to 8 mg per kilogram of body weight per day children about 5 to 7.5 mg, adults 0.7 to 1.6 mg, and aged people need about 3 to 5 mg of ascorbic acid per kilogram of body weight per day Pregnant and nursing women need 5 to 10 mg per kilogram of body weight per day The recommended daily allowances for ascorbic acid as established by the Food and Nutrition Board of the National Research Council will be found on page 6B

It is interesting to note the relatively high requirements of this vitamin on the weight basis compared to the daily needs of man and animals by the other vitamins The order of magnitude is about 1000 times the weight of some of the other vitamins required (Compare however, the high requirements of choline) A normal diet contains, however, adequate amounts of vitamin C

**THE GROUP OF
VITAMINS D**

THE GROUP OF VITAMINS D¹

1 Nomenclature and Survey

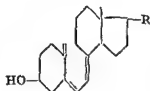
Vitamins D are usually called simply vitamin D. The individual members of this group have not been properly named as yet. Provisionally they are called vitamin D₂, D₃, D₄, etc. Vitamin D₂ is called 'calciferol' in England and has been given the name 'viosterol' by the Council on Pharmacy and Chemistry (United States).

Historical Names, now abandoned

Rachitamin
Rachitasterol²
Antirachitic vitamin

Chemical formulas

General formula for vitamins D *



Vitamin D₂
(activated ergosterol
calciferol viosterol)
R =



Vitamin D₃
(activated 7-dehydro-
cholesterol) R =



Vitamin D₄
(activated 22 dihydro-
ergosterol) R =



Vitamin D₅
(activated 7 dehydro
sitosterol) R =



(* See page 342 for footnote)

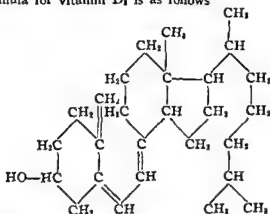
¹ See also C. I. Read, H. C. Struck and I. E. Steck, *Vitamin D*, Chicago, 1939.

² R. L. Jones, *Science* 68: 480 (1928).

2 Chronology

- 1822 TROUSSEAU in his book on *Clinical Medicine* recommended cod liver oil as a remedy for the cure of rickets¹ and J SNIADOCKI inferred in his book *On the Physical Education of Children* the curative effect of sunlight² but their view points did not become widely known
- 1890 PALM³ associated through geographical studies the incidence of rickets with deficiency of sunlight
- 1905 BUCHHOLZ⁴ apparently cured cases of human rickets with artificial light
- 1906 HOPKINS⁵ suggested that rickets is caused by the absence of an "accessory food stuff" FUNK⁶ (1914) corroborated this concept of the origin of rickets
- 1913 RACZYNSKI⁷ established the beneficial influence of sunlight on the calcium assimilation of puppies
- 1919 MELLANBY⁸ discovered the nutritional importance of animal fats for the normal calcification of bones by raising dogs affected with rickets through special diet and by curing the animals with animal fats He concluded that the anti rachitic factor⁹ is either identical with fat soluble A (vitamin A) or has a natural distribution somewhat similar to fat soluble A
- HULDSCHINSKY¹¹ proved on the basis of x ray studies that severe rickets in children can be cured by the light of a mercury vapor quartz lamp Not until this report was published was the importance of sunlight appreciated
- 1920-1921 SHERMAN and PAPPENHEIMER¹² and McCOLLUM and SIMMONDS¹³ succeeded in inducing rickets in rats by special diet

* The formulas of the vitamins D as indicated on p 341, are abbreviated The complete chemical formula for vitamin D₁ is as follows



¹ A Trousseau *Clinical Medicine* Philadelphia 1882

² W Mozolowski *Nature* 143 121 (1930)

³ T A Palm *Practitioner* 45 271 321 (1890)

⁴ E A Park *Physiol Rev* 3 166 (1923)

⁵ F G Hopkins *Analyst* 31 380 (1906) *J Physiol* 44 425 (1912)

⁶ C Funk *Die Vitamine* Wiesbaden 1914

⁷ J Raczyński *Compt rend assoc intern pediat* 1913 308

⁸ B Mellanby *J Physiol* 52 LIII (1919) *Lancet* 1 407 (1919)

¹¹ K Huldshinsky *Deut med Wochschr* 45 712 (1919) *Z orthop Chir* 39 426 (1919-20)

¹² H C Sherman and A M Pappenheimer *Proc Soc Exptl Biol Med* 18 193 (1920-21) *J Exptl Med* 34 189 (1921)

¹³ E V McCollum and N Simmonds *J Biol Chem* 47 111 139 175 207 235 507 (1921) See also V Korenchevsky *Brit Med J* 547 (1921) *Special Rept Sci Med Russia to Council No 71* (1922)

- 1921 HESS and UNGER¹⁴ proved that the earlier observations concerning the curative effect of sunlight on patients with rickets were correct. They also reported the rickets preventive effect of sunlight for rats on a rickets producing diet.
- 1922 ZUCKER, PAPPENHEIMER and BARNETT recognized that the unsaponifiable fraction of fish liver oils contained the antirachitic factor.¹⁵
- 1922 McCOLLUM and his co-workers¹⁶ established experimentally the distinctive properties of vitamin A and the antirachitic factor. At the same time HUMM¹⁷ and independently SHEETS and FUNK¹⁸ found that light would not cure the effects brought about by an insufficiency of vitamin A.
- HESS and GUTMAN¹⁹ expressed the opinion that the curative effect brought about on patients with rickets by either cod liver oil or light was fundamentally the same.
- 1924 STEENBOCK and associates²⁰ and independently HESS²¹ found that antirachitic potency could be induced in foods by ultraviolet irradiation.
- 1925 The work of HESS, WEINSTOCK and HELMAN²² of STEENBOCK and BLACK²³ and of ROSENHEIM and WEBSTER²⁴ indicated that the sterol fraction of foodstuffs could be made antirachitically active by irradiation although it was not active in itself.
- McCOLLUM named the antirachitic material vitamin D.
- 1925-1926 SCHLUTZ and MORSE²⁵ postulated the possibility and ROSENHEIM and WEBSTER²⁴, HEILBRON, KAMM and MORTON²⁷ and POHL²⁸ proved that an impurity present in ordinary cholesterol and phytosterols is responsible for the antirachitic efficacy after irradiation.
- 1927 POHL²⁹, WINDAUS and HESS³⁰ and ROSENHEIM and WEBSTER³¹ concluded from physical chemical and biological studies that the impurity in sterols or the provitamin D is ergosterol or a sterol of similar constitution such as a hypothetical dehydro-cholesterol. Ergosterol had first been isolated by BRACONNOT in 1811 and was rediscovered in 1889 by TANRET.³²

¹⁴ A. F. Hess and L. J. Unger *Proc Soc Exptl Biol Med* 18: 298 (1921).

¹⁵ T. F. Zucker, A. M. Pappenheimer and M. Barnett *Ibid* 19: 167 (1922).

¹⁶ E. V. McCollum, N. S. Simmonds, P. G. Shipley and E. A. Park *Ibid* 18: 275 (1921). *J Biol Chem* 30: 5 (1922).

¹⁷ E. M. Humm *Lancet* II: 1318 (1922).

¹⁸ O. Sheets and C. Funk *Proc Soc Exptl Biol Med* 20: 80 (1922).

¹⁹ A. F. Hess and M. G. Gutman *J Am Med Assoc* 78: 29 (1922).

²⁰ H. Steenbock *Science* 60: 274 (1924). H. Steenbock and A. Black *J Biol Chem* 61: 405 (1924). H. Steenbock and M. T. Nelson *Ibid* 62: 209 (1924).

²¹ A. F. Hess *Am J Diseases Children* 28: 517 (1924). A. F. Hess and M. Weinstock *J Biol Chem* 62: 301 (1924).

²² A. F. Hess, M. Weinstock and F. D. Helman *Ibid* 63: 303 (1925).

²³ H. Steenbock and A. Black *Ibid* 64: 263 (1925).

²⁴ O. Rosenheim and T. A. Webster *Lancet* I: 10: 5 (1925).

²⁵ F. W. Schlutz and M. Morse *Am J Diseases Children* 30: 199 (1925).

²⁶ O. Rosenheim and T. A. Webster *J Soc Chem Ind* 43: 932 (1926). *Biochem J* 21: 127 (1927).

²⁷ I. M. Heilbron, E. D. Kamm and R. A. Morton *J Soc Chem Ind* 45: 93 (1926). *Biochem J* 21: 78 (1927).

²⁸ R. Pohl *Nachr Ges Wiss Göttingen Math phys k Klasse* III, 142 (1926).

²⁹ R. Pohl *Ibid* III: 185 (1927).

³⁰ A. Windaus and A. Hess *Ibid* III: 175 (1927). A. Windaus *Ibid* III: 183 (1926).

³¹ O. Rosenheim and T. A. Webster *Lancet* I: 306 (1927). *Biochem J* 21: 389 (1927).

³² The name ergosterol originates from ergot, a black fungus which grows on the rye plant and from which ergosterol was first isolated. C. Tanret *Compt rend* 108: 95 (1889). *Ann chim phys* (VI) 20: 289 (1890). *Compt rend* 147: 75 (1908). *Ann chim phys* (VIII) 15: 313 (1908).

- 1929-1931 REERINK and VAN WIJK¹¹ isolated for the first time a crystallized vitamin D preparation made from activated ergosterol LINSERT¹² isolated the pure compound
- 1930 MUSSEHL and ACKERSON¹³ and independently MASSENGALE and NUSSMEIER¹⁴ showed that vitamin D obtained from ergosterol was not active for chicks when fed on the basis of Rat Units in amounts equal to those effective for chicks from cod liver oil
- 1933 WINDAUS and LANGER¹⁵ prepared a new synthetic provitamin D 22 dihydro ergosterol from ergosterol
- 1934 BILLS MASSENGALE and IMBODEN¹⁶ showed that the vitamin D of fish oils is not a single substance since tuna liver oil was less antirachitic than cod liver oil Rat Unit for Rat Unit in chickens
WADDELL¹⁷ found that crude cholesterol after activation yielded a vitamin D which is as effective for chicks as cod liver oil fed in equivalent Rat Units Thus the provitamin present in cholesterol must be different from ergosterol
- 1935 WINDAUS LETTRÉ and SCHENCK¹⁸ synthesized the hypothetical natural provitamin D 7 dehydro cholesterol from cholesterol
- 1936 BOER REERINK VAN WIJK and VAN NIEKERK¹¹ and later (1937) also WINDAUS and BOCK¹⁹ isolated 7 dehydro-cholesterol from cholesterol (obtained from hog skin) BROCKMANN²⁰ SIMONS and ZUCKER²¹ and HASLEWOOD and DRUMMOND²² isolated the vitamin D from tuna and from halibut liver oils in the form of crystallized esters and proved that the vitamin itself is mainly or entirely activated 7 dehydro cholesterol (vitamin D₂)
- 1937 SCHENCK¹⁸ obtained crystallized vitamin D₂ prepared by activation of 7 dehydro-cholesterol
- 1938 BILLS MASSENGALE HICKMAN and GRAY²³ isolated a new vitamin D of low biological activity by molecular distillation of cod liver oil

THE CONCEPT OF PROVITAMINS D AND OF VITAMINS D

Compounds of the physiological efficacy of vitamins D occur only in the animal organism Plants contain materials which can be converted

¹¹ E H Reerink and A van Wijk *Biochem J* 23 1794 (1929) 25 1001 (1931)

¹² O Linsert Annotation in *Ann* 489 269 (1931) A Windaus O Linsert A Löttinghaus and G Weidlich *Ibid* 492 226 (1932)

¹³ F E Mussehl and C W Ackerson *Po ltry Sci* 9 334 (1930)

¹⁴ O N Massengale and M Nussmeier *J Biol Chem* 87 423 (1930)

¹⁵ A Windaus and R Langer *Ann* 508 105 (1933)

¹⁶ C E Bills O N Massengale and M Imboden *Science* 80 596 (1934)

¹⁷ J Waddell *J Biol Chem* 105 711 (1934)

¹⁸ A Windaus H Lettré and F Schenck *Ann* 520 98 (1935)

¹⁹ A G Boer E H Reerink A van Wijk and J van Niekerk *Proc Acad Sci Amsterdam* 39 622 (1936)

²⁰ A Windaus and F Bock *Z physiol Chem* 245 168 (1937)

²¹ H Brockmann *Ibid* 241 104 (1936) *Ibid* 245 96 (1937) H Brockmann and A Busse *Ibid* 249 176 (1937)

²² E J H Simons and T F Zucker *J Am Chem Soc* 58 965 (1936)

²³ G A D Haslewood and J C Drummond *J Soc Chem Ind* 55 598 (1936)

²⁴ F Schenck *Naturwissenschaften* 25 159 (1937)

²⁵ C E Bills O N Massengale K C D Hickman and E L Gray *J Biol Chem* 126 241 (1938)

into vitamins D. These are called "provitamins D". There occur in nature a number of provitamins D and of vitamins D. The vitamins D differ in their antirachitic effectiveness in various animals. The number of known naturally occurring provitamins D and vitamins D is small. The provitamins ergosterol and 7 dehydro cholesterol and the corresponding vitamins, namely, vitamin D₂ which is also known as 'activated ergosterol' viosterol or calciferol and vitamin D₃ which is activated 7-dehydro cholesterol. Two additional provitamins D have been claimed patentwise to occur in invertebrata but very little is known about them. A number of other provitamins D and vitamins D have been prepared in the laboratory and it is suspected that some of these compounds may also occur in nature. Furthermore the existence of two more naturally occurring vitamins D is indicated on the basis of their outstanding physiological properties.

PROVITAMINS D

A provitamin D is defined as a compound that can be activated to a vitamin D. Provitamins D are compounds of the cyclopentanoperhydrophenanthrene skeleton and belong to the sterol family. They are specifically characterized by a hydroxyl group in the 3 position and a system of conjugated double bonds in ring B of the steroid nucleus, namely, in the 5,6 and 7,8 positions. Provitamins D cannot be defined physiologically but it is suspected that it will be shown eventually that those steroids of the above classification which after activation are potent vitamins D for a specific species are absorbed in the intestinal tract of an animal of that species.

According to this definition several compounds have been tentatively classified as provitamins D. They will be discussed in the following sections. A number of other compounds which fulfill only partly the definition of provitamins as given above are discussed under 'Specificity of Vitamin D' (page 406).

3 Occurrence

Provitamins D are widely distributed over the animal and plant kingdom. While it is impossible to make definite statements as to what provitamin D occurs in various specific natural sources, certain generalizations can be made. It appears that the most prevalent provitamin D in higher animals and in human beings is 7 dehydro-cholesterol. Plants, molds and

yeast contain predominantly ergosterol⁴⁸ Considerable uncertainty exists about the kind of provitamins D in lower animals Thus, ergosterol occurs in the snail *Arion empiricorum* and in the earthworm, and 7 dehydro cholesterol in the snail *Buccinum undatum*⁴⁹ Mussels⁵⁰ are said to contain a different provitamin D and the same has been claimed for periwinkles⁵¹

The provitamin D content in different sources varies considerably Thus in higher animals, the provitamin D content is the greatest in the skin, namely, about 4% of the total sterol content, whereas the sterols from the inner organs contain only from 0.1 to 0.5% provitamin D (This is due to the activation mechanism of provitamins D to vitamins D as will be explained later) The best sources of ergosterol are yeast and certain molds some of which contain this provitamin as practically the only sterol The highest concentration of 7 dehydro cholesterol has been found in a species of snails (*Buccinum undatum*) and is 27% of the total sterol fraction The following tables indicate, as far as is known, the content of ergosterol and of 7 dehydro cholesterol in the sterol fraction of various sources Since in most naturally occurring materials the type of provitamin is not known another table shows the general provitamin D content in sterols from various materials

TABLE I
ERGOSTEROL CONTENT OF VARIOUS MATERIALS

Source	Provitamin D in sterols %
Dried yeast	90-100
Snail <i>Arion empiricorum</i>	19-25
Earthworm	22
Cottonseed oil	5
Scopolia root	1.4

TABLE II
7 DEHYDRO CHOLESTEROL CONTENT OF VARIOUS MATERIALS

Source	Provitamin D in sterols %
Pigskin	3-6
Snail <i>Buccinum undatum</i>	17-27

⁴⁸ C. Taurin, *Compt. rend.* 108, 98 (1889); *Ann. chim. phys.* (VI) 20, 289 (1890); *Compt. rend.* 147, 75 (1908); *Ann. chim. phys.* (VIII) 13, 313 (1908).

⁴⁹ A. Windaus, *Vierteljahrsschrift der Naturforschenden Gesellschaft in Basel* 33, 185 (1938); F. Bock and F. Wetter, *Z. physiol. Chem.* 256, 33 (1938).

⁵⁰ A. G. Boer, J. van Niekerk, E. H. Reerink, and A. van Wijk, *U.S.P.* 2,163,659.

⁵¹ A. G. Boer, J. van Niekerk, E. H. Reerink, and A. van Wijk, *U.S.P.* 2,216,719.

TABLE III
PROVITAMIN D CONTENT OF VARIOUS MATERIALS

Source	Provitamin D in sterols %
Vertebrata	
Skin from man	0 15-0 43
cattle	0 18
calf	0 68
mice	0 87
chicken feet ^{51a}	1 0-4 0
chicken trunk ⁵¹	0 001-0 01
Blood serum (cow)	0 15
Brain (cow)	0 01
Lung (calf)	0 025
Heart (calf)	0 032
Spleen (cow)	0 045
Placenta (cow)	0 18
Pancreas (cattle)	0 18
Invertebrata	
Lugworm (<i>Arenicola marina</i>)	4-12
Mussels (<i>Mytilus edulis</i>)	9-10
Oysters	5-6
Leech	4
Crabs (<i>Cancer pagurus</i>)	0 32
Sea anemones	2-10
Plants	
Cocksfoot grass	0 80
Rye grass	1 5
Wheat germ oil	0 8
Seaweed	0 008
Cabbage	0 05
Spinach	1 0
String beans	0 1

4 Isolation

The isolation of provitamins D from natural sources involves two different steps namely the isolation of the total sterols and the separation of the provitamins from other sterols present. The isolation of the total sterols is usually a simple process and consists in either first extracting

⁵¹ H. R. Rosenberg unpublished data.

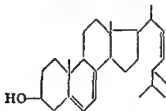
the total fat, followed by saponification of the fatty material and isolation of the unsaponifiable fraction, or in saponifying directly the total material followed by isolation of the non saponifiable fraction. The isolation of the sterols from the non saponifiable fraction is usually carried out by crystallization from a suitable solvent, such as alcohol. Special methods have been recommended for special cases. For example, after saponification, the fatty acids may be precipitated as calcium salts which adsorb the sterols. These are then recovered by solvent extraction from the filtered precipitate. Another modification has been used in those cases where the total amount of fatty acids is very low. Sodium benzoate is added to the saponification mass and the entire mixture is acidified. Benzoic acid precipitates and adsorbs the sterols present which can then easily be isolated by alkaline extraction of the benzoic acid.

The separation of the provitamins from other sterols is usually a difficult problem and success depends largely upon the type and amount of provitamin D present. Thus no method has been found by which the provitamin D present in cholesterol from the spinal cord of cattle can be isolated satisfactorily, since the provitamin D content is only 0.1%. Better chances for a successful isolation exist when the provitamin D is present in a concentration of at least 4-5% or more. The usual method is fractional adsorption of the sterols or of their esters, for example, on aluminum oxide, which in many cases permits an almost quantitative separation. If these methods fail, a condensation product with maleic or citraconic acid anhydride may be formed which can be split by thermal decomposition into the provitamin D and the acid anhydride.

5 Properties

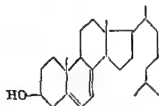
The following provitamins D are known

- (1) **Ergosterol** Ergosterol crystallizes in small colorless crystals with water of crystallization. The melting point varies according to the degree of hydration. The best crystallized preparation contains $1\frac{1}{2}$ mols of water and melts at 168° C. Complete dehydration is very difficult to achieve and results in a product with a melting range from 166 to 183° C. Ergosterol distills in high vacuum at 250° C without decomposition. $[\alpha]_D^{20} = -130$ (-135°) and $[\alpha]_{5461}^{20} = -171^\circ$ (in chloroform).



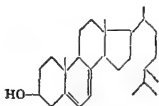
- (2) **Epi-ergosterol** Physical constants unknown

- (3) 7-Dehydro-cholesterol M p 150-151° C ⁴⁴ $[\alpha]_D^{20} = -113.6$ in chloroform



- (4) Epi 7 dehydro-cholesterol ⁴⁴ M p 124-126° C $[\alpha]_D^{20} = -70.5$ in chloroform

- (5) 22 Dihydro-ergosterol ⁴⁴ M p 152-153 C $[\alpha]_D^{20} = -109$ in chloroform



- (6) 22 23 Oxido ergosterol ⁴⁴ Physical constants unknown

- (7) "Mussel provitamin" ⁴⁵ Constitution unknown M p 150-151 C $[\alpha]_D^{20} = -118^\circ$ in benzene

- (8) "Periwinkle provitamin" ⁴⁵ Constitution unknown M p 137-137.5 C $[\alpha]_D^{20} = -124^\circ$ in benzene

- (9) 7 Dehydro-sitosterol M p 144-145 C $[\alpha]_D^{20} = -116$ in chloroform

- (10) 7 Dehydro-stigmasterol M p 154° C $[\alpha]_D^{20} = -113.15$ in benzene

All these provitamins have similar solubility characteristics. They are insoluble in water but soluble in the typical organic solvents, such as ether, hydrocarbons, chlorinated hydrocarbons, alcohols, etc. The lower alcohols are usually used for recrystallization. The provitamins separate from the alcohols with water (or solvent) of crystallization.

All provitamins have the same characteristic absorption spectrum in the ultraviolet which is characterized by maxima at 260, 270, 281 and 293.5 μ (Fig. 15). The molecular absorption coefficient $K = \text{about } 30 \times 10^4$ for the band at 281 μ .

⁴⁴ A. C. Boer, E. H. Reerink, A. van Wijk and J. van Nierck, *Proc. Acad. Sci. Amsterdam* 39, 622 (1936).

⁴⁵ A. Windaus and J. Nagg, *ts. Ann.* 542, 204 (1939).

⁴⁶ A. Windaus and R. Lang, *ts. Ibid.* 508, 105 (1933).

⁴⁷ A. Windaus, Linsal and K. Buchholz, quoted by K. Dimroth and J. Feland, *Ber.* 72, 187 (1939).

⁴⁸ A. C. Boer, J. van Nierck, E. H. Reerink and A. van Wijk, *U. S. P.* 2,163,659.

⁴⁹ A. C. Boer, J. van Nierck, E. H. Reerink and A. van Wijk, *U. S. P.* 2,216,719.

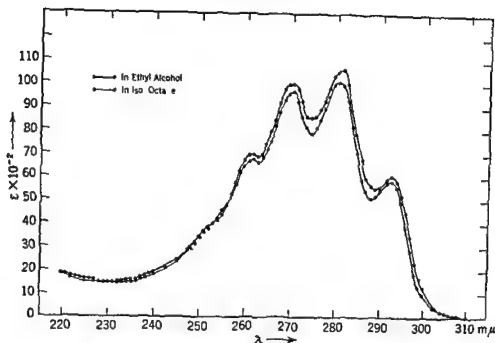


Fig 15—Absorption spectrum of ergosterol in ethanol and isooctane (T R Hogness A E Sidwell and F P Zscheile)

6 Chemical Constitution

(a) Ergosterol

Ergosterol (I) has the empirical formula $C_{28}H_{44}O$ ⁵⁹ which was established by careful analysis of derivatives containing hetero atoms, such as the 3,5 dinitro benzoate halogeno nitro benzoates etc. The oxygen is present in a hydroxyl group, since esters can be obtained with acid anhydrides or acid chlorides in the presence of an amine. Ergosterol contains three double bonds, since upon catalytic hydrogenation six atoms of hydrogen are absorbed⁶⁰. The totally saturated compound is called ergostanol (II) and has the formula $C_{28}H_{50}O$. It follows that four ring systems are present. From ergostanol the corresponding hydrocarbon ergostane (IV) can be obtained by conversion into ergostanyl chloride (III) followed by reduction with sodium and amyl alcohol⁶¹. Ergostane upon oxidation with chromic acid, yields a mono carboxylic acid, $C_{28}H_{48}O_2$ ⁶² which is identical with the nor allo cholanolic acid (V) obtained from cholesterol

⁵⁹ A Windaus and A Lüttringhaus *Nachr Ges Wiss Göttingen Math physik Klasse III* 4 (1932)

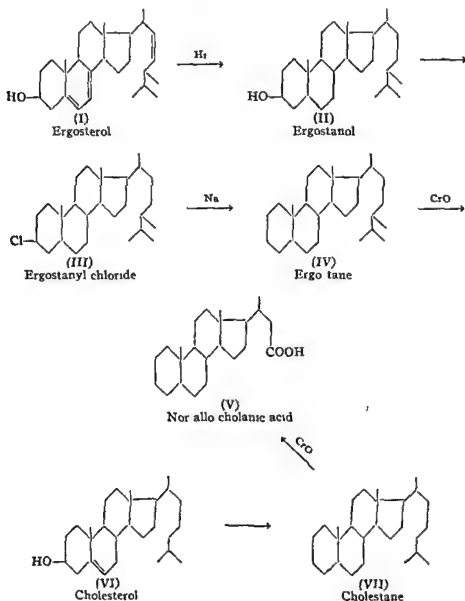
A Windaus F v Werder and E Gschäuder *Ber* 65 1008 (1932)

⁶⁰ A Windaus and O Linser *Ann* 465 154 (1928)

⁶¹ F Reindel and E Walter *Ibid* 460 222 (1928)

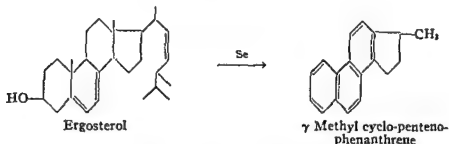
⁶² C K Chuang *Ibid* 500 270 (1933)

(VI) *via* cholestane (VII) This degradation reaction proves that ergosterol belongs to the class of sterols, which are characterized by the cyclopentano perhydro phenanthrene skeleton. Furthermore it follows that the steric configuration of cholestane and that of ergostane are the same

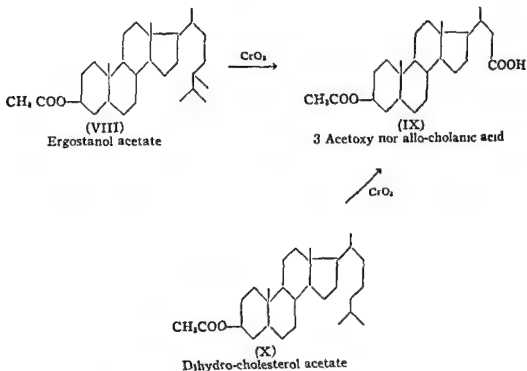


That ergosterol belongs to the sterols has furthermore been demonstrated since it yields upon total dehydrogenation with selenium

γ methyl cyclo penteno phenanthrene ⁴³ which is the typical dehydrogenation product of all sterols. No other class of compounds yields this particular hydrocarbon upon dehydrogenation.



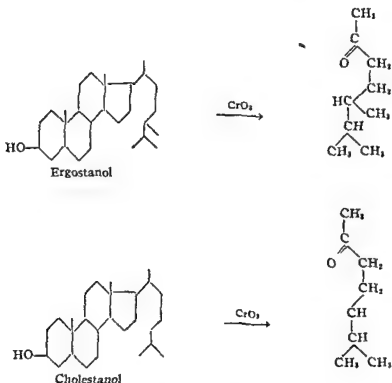
The position of the hydroxyl group in the ring system was shown to be at carbon atom 3 ⁴⁴ since acetylated ergosterol (VIII) yielded upon chromic acid oxidation, 3 acetoxy nor allo cholanolic acid (IX) which was also obtained from dihydro cholesterol acetate (X). This result also proves that the steric configuration of the hydroxyl group is of the β type which was suspected since ergosterol can be precipitated with digitonin.



⁴³ O. Diefs and A. Karstens *Ann.* 478 129 (1930)

⁴⁴ E. Fernholz and P. N. Chakravorty *Ber.* 67 2021 (1934)

The chromic acid oxidations indicate furthermore, that the basic structure of the ring system and of the first five carbon atoms of the side chain is identical in both cholesterol and ergosterol. Since ergosterol contains one carbon atom more than cholesterol this must be located in that part of the side chain which is removed during the oxidation. This can be proved, since by energetic oxidation of ergostanol a ketone containing nine carbons ($C_9H_{18}O$) is obtained,⁶⁵ whereas cholestanol under the same conditions yields a ketone of only eight carbon atoms

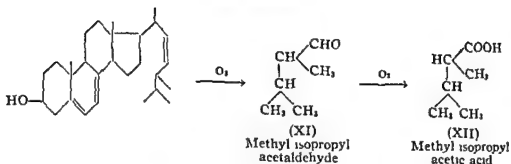


Ergosterol upon ozonization yields methyl isopropyl acetaldehyde^{66 67} (XI) which was identified by the formation of its semicarbazone and di nitro phenyl hydrazone and by oxidation to methyl isopropyl acetic acid (XII). This result indicates that a double bond is in the side chain between carbon atoms 22 and 23.

⁶⁵ A. Guiteras, *Ann.* 494 116 (1932)

⁶⁶ F. Reindel and H. Kipphan, *Ibid.* 493 181 (1932)

⁶⁷ A. Guiteras, *Ibid.* 494 116 (1932)

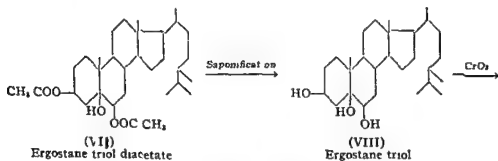
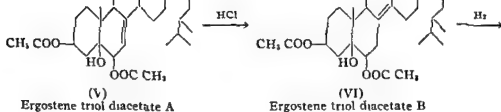
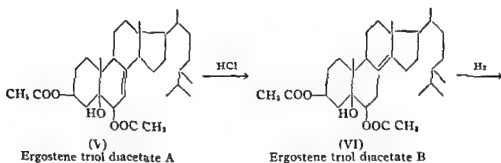
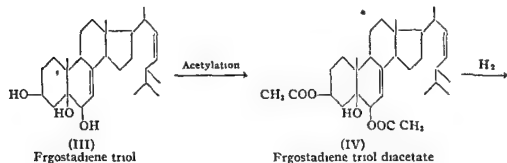
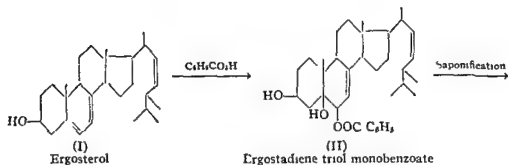


Another double bond is present in the 5 6 position which is the position of the double linkage in cholesterol. This has been proved by the following series of reactions.⁶⁸ Ergosterol (I) upon addition of perbenzoic acid to one of its double bonds yields an ergostadiene triol monobenzoate (II)⁶⁹ which contains one free secondary and one free tertiary hydroxyl group since only one of these can be readily esterified. By saponification of this product followed by acetylation an ergostadiene triol diacetate (IV) is thus formed. The diacetate upon catalytic hydrogenation takes up only one mol of hydrogen (forming V) and leaving one other double bond in the molecule. This is rearranged by the action of hydrochloric acid. The newly formed compound (VI) takes up hydrogen easily,⁷⁰ yielding the totally hydrogenated ergostane triol diacetate (VII) which upon saponification gives the free ergostane triol (VIII). Two of the hydroxyl groups in this compound are in $\alpha \beta$ position to each other since upon oxidation with lead tetra acetate according to Criegée one atom of oxygen is consumed. By chromic acid oxidation of the triol a hydroxy diketone (IX) is formed which splits out water by the action of hydrochloric acid. The unsaturated diketone ergostene dione (X), can be transformed into a saturated diketone ergostadione (XI), by means of zinc and acetic acid. Hydrazine condenses readily with the diketone with the formation of a pyridazine derivative (XII). The hydroxyl group originally present in ergosterol is in 3 position and therefore one of the keto groups in ergostadione is in 3 position. Since the other keto group according to its reactions must be three carbon atoms removed from the keto group at carbon atom 3, it can only be located at carbon atom 6. The diketone is, therefore ergostadione 3 6. It follows furthermore, that the tertiary hydroxyl group in the ergostane triol is in 5 position the triol thus having the constitution of a 3,5 6 triol.

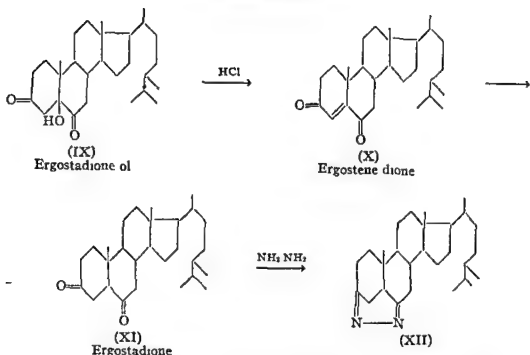
⁶⁸ A. Windaus, H. H. Inhoffen and S. v. Reichel, *Ann.* 510 248 (1934).

⁶⁹ A. Windaus and A. Luttringhaus, *Ibid.* 481 127 (1930).

⁷⁰ I. M. Heilbron, A. L. Morrison and J. C. E. Simpson, *J. Chem. Soc.* 1933 302.



(Formula continued on following page)



The position of the third double bond is established by the refractive index⁷¹ by x ray measurements,⁷² and by the ultraviolet absorption spectrum all of which exhibit characteristics of a system of two conjugated double bonds. The last double linkage cannot be located in the side chain due to the degradation reactions discussed above but must be in conjugation to the double bond in the 5,6 position. Thus the only possible position is the 7,8 position. The conjugation of the double bonds is furthermore suspected by the fact that ergosterol can be reduced with sodium and amyl alcohol⁷³ and by the fact that ergosterol forms a characteristic addition product with maleic anhydride⁷⁴ from which ergosterol can be recovered by thermal decomposition.⁷⁵ Since it is known that one ring double bond is in the 5,6 position, this maleic anhydride condensation is only possible when the other double bond conjugated with the one in the 5,6 position is located in the same ring in which the first double bond is located. Further proof is indicated by nitric acid oxidation of ergosterol which yields toluene 2,3,4,5 tetracarboxylic acid.⁶ While the value of this reaction by itself is limited since a migration of a methyl group is

⁷¹ K. v. Auwers and E. Wolter, *Vierteljahrsschrift der Naturforschenden Gesellschaft in Zürich* 26, 101 (1931).

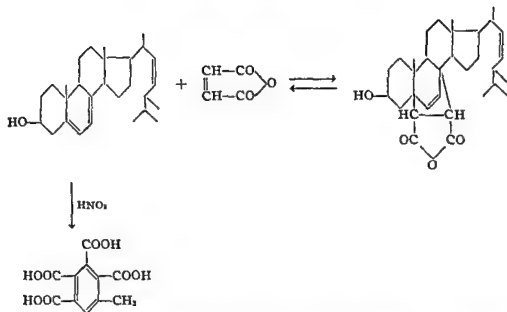
⁷² G. E. R. Schulze, *Z. physik. Chem.* A171, 436 (1934).

⁷³ A. Windaus and J. Brunken, *Ann.* 460, 275 (1928).

⁷⁴ A. Windaus and A. Luttringhaus, *Ber.* 64, 850 (1931).

⁷⁵ H. H. Inhoffen, *Ann.* 508, 81 (1933).

⁷⁶ F. Reindel and K. Niederländer, *Ibid.* 482, 264 (1930); H. H. Inhoffen, *Ibid.* 494, 122 (1932).



involved the result is corroborated by another series of reactions. Ergosterol is dehydrogenated by eosin in the absence of oxygen when exposed to visible light with the formation of a bimolecular compound,⁷⁷ in which two molecules are linked together through the 7 7' position with a shift of the 7 8 double bond into the 8 9 position⁷⁸ (II). Upon thermal decomposition of this compound methane is evolved⁷⁹ and neoergosterol (III) is formed⁷⁷⁻⁸⁰ which contains an aromatic ring as indicated by the absorption spectrum and by the fact that only one double bond can be detected by perbenzoic acid or by catalytic hydrogenation. That this double bond is in the side chain is proved by ozonization of neoergosterol which yields methyl isopropyl acetaldehyde.⁸¹ The reaction of nitric acid on neoergosterol yields mellophanic acid (benzene 1,2,3,4-tetracarboxylic acid (IV)) which differs from the nitric acid oxidation product of ergosterol by the absence of a methyl group. While these results can be explained only by the assumption that ring B is aromatic, further proof has been brought forward. The hydroxyl group of neoergosterol is aliphatic in character, not phenolic, thus excluding the possibility that ring A has become aromatic. On the other hand, upon catalytic dehydrogenation with platinum a β -naphthol derivative, dehydro neoergosterol (V), is

⁷⁷ A. Windaus and P. Borgeaud, *Ann.* 460, 235 (1928).

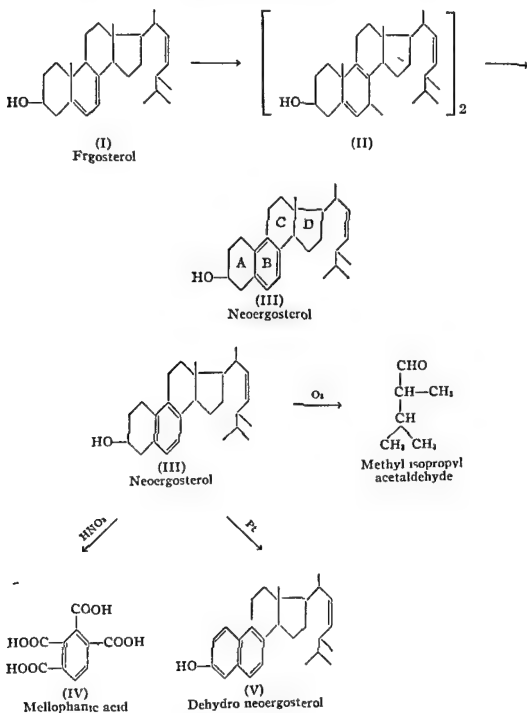
⁷⁸ H. H. Inhoffen, *Naturwissenschaften* 25, 125 (1937).

⁷⁹ H. H. Inhoffen, *Ann.* 497, 130 (1932).

⁸⁰ K. Bonstedt, *Z. physiol. Chem.* 185, 165 (1929).

⁸¹ H. H. Inhoffen, *Ann.* 497, 130 (1932).

obtained⁸ This result excludes the possibility that ring C in neoergosterol is aromatic in character



⁸ H. Honigsmann, *Ann.* 511, 292 (1934)

Ergosterol is a very sensitive compound. Acids cause rearrangements of the double bonds, oxygen brings about the formation of peroxides and hydrogen causes the formation of a number of different di- and polyhydro compounds.

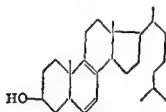
(b) *The Other Provitamins D*

The proof for the chemical constitution of the other provitamins D is mainly based upon the determination of the characteristic absorption spectrum which is common to all provitamins D since they all have the same system of conjugated double bonds in ring B. The constitution of the provitamins 7 dehydro cholesterol and 22 dihydro ergosterol is further more established since these compounds have been obtained by synthetic methods from cholesterol and from ergosterol respectively. The constitution of the epi compounds, namely of epi ergosterol and of epi 7 dehydro cholesterol is established since these provitamins do not precipitate with digitonin whereas the parent provitamins ergosterol and 7 dehydro cholesterol form addition compounds with digitonin. This reaction in combination with their absorption spectrum and the method of synthesis of the epi compounds proves that they differ from their parent provitamins D only in the stereochemical configuration of the hydroxyl group in 3 position. This position of the 3 hydroxyl group must be related to some other group in the sterol ring system. Ruzicka chose the relationship between the substituents on C₃ and on C₆ as reference for the *cis trans* isomerism of the 3 hydroxyl group. Since the provitamins D have no hydrogen at C₆, this system applies by implication. Ergosterol, 7 dehydro cholesterol etc. belong according to this nomenclature to the *trans* derivatives whereas the compounds of the epi family which do not precipitate with digitonin belong to the *cis* derivatives. Another system of nomenclature suggested by Schoenheimer uses the relation of the hydroxyl group in 3 position to the methyl group on carbon atom 10. According to this system ergosterol and 7 dehydro cholesterol belong to the *cis* C₃ C₁₀ compounds and the epi derivatives belong to the *trans* C₃ C₁₀ compounds.

The constitution of the mussel provitamin D and of the periwinkle provitamin D are unknown. It has tentatively been assumed that the mussel provitamin D has 29 carbon atoms⁴³ that is it has one carbon atom more than ergosterol or two carbon atoms more than 7 dehydro cholesterol.

⁴³ A. G. Boer, J. van N'ekerk, E. H. Reerink and A. van Wijk, U. S. P. 2,163,659.

benzoates⁹⁴ or of substituted benzoates⁹⁵ Isodehydro cholesterol has the probable formula (VIII),⁹³ that is, it differs from 7 dehydro cholesterol only in the position of the double bonds, which are believed to be in the 6,7 and 8,9 positions



(VIII)

Isodehydro-cholesterol

Besides this clear cut synthesis a number of other methods have been found to yield 7 dehydro cholesterol in small amounts. Thus by direct oxidation of cholesterol under mild conditions⁹⁶ for example, with per oxides, a provitamin D is obtained which is probably 7 dehydro cholesterol. Somewhat higher yields are apparently obtained by the utilization of quinones as dehydrogenating agents^{97, 98}. Also methylene blue in the presence of light, and succino dehydrogenase have been claimed to convert cholesterol into provitamin D⁹⁹.

A number of other provitamins D are claimed to have been obtained from cholesterol or its derivatives. The following methods used for their preparation indicate that the provitamins obtained may ultimately prove to be 7 dehydro cholesterol, although some investigators believe them to be new and different provitamins D¹⁰⁰.

(a) Cholesterol upon heating, is converted in very small amounts into a provitamin D¹⁰¹.

(b) Cholesterol freed essentially from its naturally adherent provitamin D content and subjected to ultraviolet light, yields a vitamin D¹⁰².

⁹⁴ A. G. Boer, E. H. Reerink, A. van Wijk and J. van Niekerk *Proc. Acad. Sci. Amsterdam* 39 622 (1936).

⁹⁵ A. Windaus, O. Linsert and H. J. Eckhardt *Ann.* 534 22 (1938).

⁹⁶ J. Waddell *U. S. P.* 2 028 364; *U. S. P.* 2 056 992.

⁹⁷ N. A. Milas and R. Heggie *J. Am. Chem. Soc.* 60 984 (1938).

⁹⁸ P. I. T. Sab *Rec. Trav. chim.* 59 404 (1940).

⁹⁹ N. A. Milas and R. Heggie *J. Am. Chem. Soc.* 60 984 (1938).

¹⁰⁰ C. E. Bills *Cold Spring Harbor Symposia Quant. Biol.* 3 328 (1935).

¹⁰¹ E. M. Koch and F. C. Koch *Science* 82 394 (1935); *J. Biol. Chem.* 116 756 (1936); M. L. Hathaway and D. E. Lobb *Ibid.* 113 105 (1936); R. W. Hamann and H. Steenbock *Ibid.* 114 505 (1936).

¹⁰² C. E. Bills *J. Biol. Chem.* 66 451 (1925); A. Jendraszyk and A. G. Keményfi *Biochem. Z.* 189 180 (1927); S. K. Kou, F. Daniels and H. Steenbock *J. Am. Chem. Soc.* 50 2573 (1929); F. C. Koch, E. M. Koch and J. K. Ragins *J. Biol. Chem.* 85 141 (1929); E. M. Koch and H. B. Lemon *Ibid.* 85, 159 (1929).

This vitamin D is alleged to be different from that obtained from 7 dehydro cholesterol since it was found to be less effective for chicks

(c) 7 Hydroxy cholesterol upon irradiation develops a slight anti rachitic potency¹⁰³ The work does not indicate whether or not pure 7-hydroxy cholesterol was employed There exists the possibility that 7 hydroxy cholesterol can lose one mol of water to form 7 dehydro cholesterol

(d) 7 Oxo cholesterol acetate upon Grignard reaction with isobutyl magnesium bromide followed by heating to 200° C is said¹⁰⁴ to produce small amounts of a new provitamin D While this statement has not been proved, it appears possible that the Grignard compound led to a partial reduction to a 7 hydroxy cholesterol compound Such reductions have been observed to occur in sterols¹⁰⁵ 7 Hydroxy cholesterol may then upon heating be transformed partially into 7 dehydro cholesterol

(b) *The Synthesis of Epi 7-dehydro cholesterol*

The synthesis of epi 7 dehydro cholesterol has been carried out (1) by using epi cholesterol as starting material¹⁰⁶ and employing the methods outlined for the synthesis of 7 dehydro cholesterol and (2) by epimerization of the 3 hydroxyl group of 7 dehydro cholesterol¹⁰⁷ The latter compound upon oxidation with aluminum *tert* butyroxide yields a dehydro cholestenone which upon reduction with aluminum isopropoxide is converted into epi 7 dehydro cholesterol in a yield of 1.25%

(c) *The Synthesis of Epi ergosterol*

Epi ergosterol has been obtained in about 1.3% yield by reduction of ergosterone with aluminum isopropoxide but has not been isolated in a high state of purity¹⁰⁸

(d) *The Synthesis of 22 Dihydro ergosterol*

22 Dihydro ergosterol has been obtained by side chain hydrogenation of ergosterol¹⁰⁹ This is carried out by acetylating ergosterol followed by formation of an addition product of maleic anhydride with ergosterol

¹⁰³ C. E. Bills *Cold Spring Harbor Symposium Quant Biol* 3: 328 (1935)

¹⁰⁴ S. Weissman and M. S. Kharasch *J Org Chem* 1: 490 (1936)

¹⁰⁵ L. Ruzicka and H. R. Rosenberg *Helv Chim Acta* 19: 357 (1936)

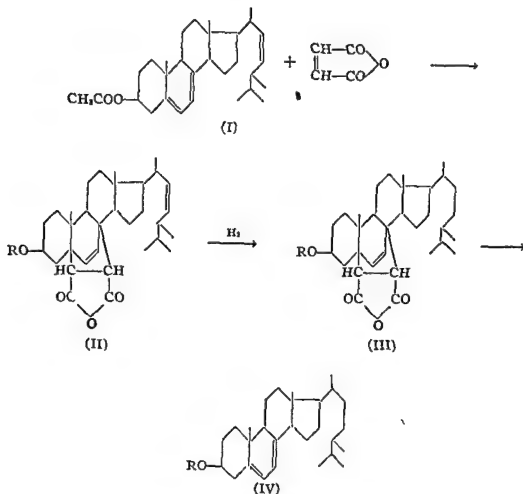
¹⁰⁶ A. Windaus and J. Nagatz *Ann* 542: 204 (1939)

¹⁰⁷ A. Windaus and O. Kaufmann *Ibid* 542: 218 (1939)

¹⁰⁸ A. Windaus and K. Buchholz *Ber* 71: 576 (1938) 72: 597 (1939)

¹⁰⁹ A. Windaus and R. Langer *Ann* 508, 105 (1933)

acetate (II) This, upon catalytic hydrogenation, is selectively reduced in the side chain (III) By thermal decomposition, 22 dihydro ergosterol acetate (IV) is obtained This yields 22 dihydro ergosterol upon saponification



(e) *Synthesis of 22 23 Oxido ergosterol*

22 23 Oxido ergosterol has been mentioned in the literature¹¹⁰ but the method of synthesis has not been reported as yet. Probably the maleic anhydride addition product of an ergosterol ester is treated with a mild oxidizing agent to form the 22 23 oxido compound which upon thermal decomposition yields the 22 23 oxido ergosterol ester.

¹¹⁰ A. Windaus, I. Ince and K. Buchholz, quoted by K. Dimroth and J. Paland, *Ber.* 72, 187 (1939).

(f) *Synthesis of 7 Dehydro sitosterol*

The synthesis of 7 dehydro sitosterol has been carried out¹¹¹ according to the Windaus method for the synthesis of 7 dehydro cholesterol. As starting material the sitosterol mixture from soybean oil was used. This contains various isomers which are extremely difficult to separate.

(g) *Synthesis of 7 Dehydro stigmasterol*

7 Dehydro stigmasterol has been synthesized¹¹² from stigmasterol according to the previously outlined method for the synthesis of 7 dehydro cholesterol from cholesterol.

8 Industrial Methods of Preparation

The industrial preparation of the provitamins ergosterol and 7 dehydro cholesterol is of technical importance. The provitamin D from certain marine animals is also used commercially. Ergosterol is manufactured mainly by extraction from yeast and to a small extent also from the mycelium of *aspergillus niger*. Commercial ergosterol contains as much as 5% of α dihydro ergosterol¹¹³ which accompanies ergosterol in fungi and which has about the same solubility characteristics as ergosterol. Extensive investigations on the ergosterol content of yeast revealed that the yields are more dependent upon the conditions under which the yeast is grown than upon the strain of yeast.

7 Dehydro cholesterol is synthesized from cholesterol according to the methods described previously.

9 Biogenesis

Little is known about the biogenesis of the provitamins D. Ergosterol is synthesized by yeast and by a number of fungi and other lower organisms. This synthesis is apparently more closely related to the metabolism of carbohydrates than to that of fats¹¹⁴. On the other hand *aspergillus niger* can synthesize ergosterol when sodium acetate is the sole source of carbon^{115, 116}. No connection could be demonstrated between sterol

¹¹¹ W. Wunderlich *Z. physiol. Chem.* 241 116 (1936).

¹¹² O. Linsert *Ibid.* 241 125 (1936).

¹¹³ R. K. Callow *Biochem. J.* 31 87 (1937).

¹¹⁴ C. E. Bills *Physiol. Rev.* 15 1 (1935). A. Heidischka and H. L. Doer *Z. physiol. Chem.* 181 15 (1929).

¹¹⁵ H. H. Magusan and E. Walker *Biochem. J.* 34 804 (1940).

¹¹⁶ I. Smedley MacLean and D. Hoffert *Ibid.* 17 70 (1923). R. Soederhoff and H. Thomas *Ann.* 530 195 (1937).

synthesis and the nitrogen metabolism^{117 118} 7 Dehydro cholesterol is apparently synthesized by higher animals and by human beings. The course of this synthesis is not known, but it is perhaps significant that both forms of 7 hydroxy cholesterol the α and the β isomer, apparently occur in animal tissues. Thus the formation of 7 dehydro cholesterol by dehydration of the 7 hydroxyl compounds appears possible. It has also been postulated that 7 dehydro cholesterol is synthesized from cholesterol by enzymatic dehydrogenation. No proof for this hypothesis can be offered other than the observation that small amounts of 7 dehydro cholesterol and of dihydro cholesterol always are present with cholesterol.¹¹⁹ It has been predicated therefore that while one molecule of cholesterol is dehydrogenated another molecule is hydrogenated. On the other hand, it seems plausible to assume that in certain cells 7 dehydro cholesterol is totally synthesized in a manner similar to the total synthesis of cholesterol which has been reported as being built up from compounds of only two or three carbon atoms.^{1 0}

10 Determination

(a) Physical Methods

The most accurate method for the determination of provitamins D is the spectroscopical analysis. The characteristic absorption spectrum of the provitamins in the ultraviolet region is the same for all provitamins on a molecular basis. This method while quite accurate for the determination of provitamins D in sterol mixtures isolated from natural sources cannot distinguish between the individual provitamins D. This method can also be used for the determination of provitamins D in preparations obtained by chemical synthesis with the restriction that the actual absorption maxima must be determined as such and in relation to each other and that general absorption must be observed and properly considered. In dubious cases, the addition of known amounts of a provitamin D may increase the accuracy of the determination of the original provitamin D content.

¹¹⁷ W. H. Maguigan and E. Walker *Biochem J.* **34** 804 (1940).

¹¹⁸ O. N. Masengale, C. E. Bills and P. S. Prickett *J. Biol. Chem.* **94** 213 (1931). M. Sobotta, W. Halden and F. Bilger *Z. physiol. Chem.* **234** 1 (1935). F. Rendl, K. Niederländer and R. Pfundt *Biochem. Z.* **291** 1 (1937).

¹¹⁹ R. Schönheimer, H. v. Behring, R. Hummel and L. Schindel *Z. physiol. Chem.* **192** 73 (1930). *Naturwissenschaften* **18** 156 (1930).

¹²⁰ D. Rittenberg *J. Biol. Chem.* **119** LXXXIII (1937).

(b) *Chemical Methods*

No chemical method has been developed which is specific enough to determine the provitamins D accurately. Since all methods known are based on the characteristic conjugated unsaturation of the provitamin D molecule, no distinction can be made between the various provitamins. The chemical color tests based on this unsaturation are the following:

1 **The Reversed Salkowski Reaction**¹²¹ The provitamin D is dissolved in chloroform and concentrated sulfuric acid is added. The acid becomes deep red while the chloroform layer remains colorless. Sterols such as cholesterol and sitosterol give the opposite color reactions: the chloroform becomes red while the acid exhibits a green fluorescence.

2 **The Liebermann-Burchard Reaction**¹²² The provitamin D is dissolved in chloroform, and acetic anhydride and concentrated sulfuric acid are added dropwise. A red color develops which progressively changes from blue violet to green. Cholesterol gives a similar change of color but the initial red color remains unchanged for a longer period of time.

3 **The Tortelli-Jaffe Reaction**¹²³ A solution of provitamin D in acetic acid is mixed with a 2% solution of bromine in chloroform. A green color develops. This reaction is given by steroids with a di-tertiary ethenoid linkage¹²⁴ and by vitamins D.

4 **The Rosenheim Reaction**¹²⁵ To a solution of provitamin D in chloroform a solution of trichloroacetic acid in water is added. A red color develops which changes slowly into a light blue. This color reaction becomes considerably more sensitive when prior to the addition of the trichloroacetic acid, lead tetraacetate in glacial acetic acid is added to the chloroform solution of the provitamin. An intense green fluorescence occurs which is however not given by provitamin D esters. Thus, provitamins can be differentiated from their esters by this method. This modified color reaction can be employed for the detection of provitamins D in an amount of the order of 0.1 γ .¹²⁶

5 **Chloralhydrate Reaction** Crystals of provitamin D, when heated slowly with crystals of chloralhydrate, melt above 50° C and the

¹²¹ E. Gérard *Chem. Zentralbl.* (1895) II 229. *Arch. ges. Physiol. Pflügers* 6 207 (1872).

¹²² C. Liebermann *Ber.* 16 1804 (1883). A. Heuschka and H. Lindner *Z. physiol. Chem.* 181 15 (1929).

¹²³ E. P. Häussler and E. Brauchli *Helv. Chim. Acta* 12 187 (1929). I. M. Heibron and F. S. Spring *Biochem. J.* 24 133 (1929).

¹²⁴ U. Westphal *Ber.* 72 1243 (1939).

¹²⁵ O. Rosenheim *Biochem. J.* 23 47 (1929). 25 74 (1931).

¹²⁶ A. von Christians and V. Anger *Ber.* 72 1124 1482 (1939).

mixture becomes first red, then green and finally deep blue. Sterols, such as cholesterol, do not give any color reaction with chloralhydrate.

6 The Antimony-Trichloride Reactions¹²⁷ Provitamin D dissolved in chloroform and mixed with a solution of antimony trichloride in chloroform yields a red color.

7 The Tschugajeff Reaction¹³⁰ A solution of provitamin D in glacial acetic acid with an excess of acetyl chloride and zinc chloride yields upon heating to the boiling point an eosin red color with a greenish yellow fluorescence. The sensitivity is reported to be 1/80,000.

While none of these reactions is absolutely quantitative the Liebermann Burchard reaction and the Rosenheim reaction^{128, 129} have been recommended and developed for quantitative assays.

CONVERSION OF PROVITAMINS D TO VITAMINS D

11 Process of Activation

Provitamins D can be activated to vitamins D by a number of different processes. They all involve in principle the input of energy into the provitamin D molecule. Thus ultraviolet light, cathode rays, radium emanation etc., effect activation.

(a) Ultraviolet Light Activation

Provitamins D are activated to vitamins D by ultraviolet light of the same wave lengths as those which are absorbed by the provitamins as evident from the absorption spectrum. The energy required to produce one U. S. Pharmacopoeia unit of vitamin D from ergosterol has repeatedly been investigated.¹³¹ The data obtained under the most careful conditions indicate that 7.5×10^{13} quanta will produce one U. S. Pharmacopoeia unit of vitamin D.¹³² In the active region, the energy necessary depends upon the wave length. The most effective activation of ergosterol is obtained from light of the wave length 281 mμ, which is the line that shows maximum absorption of ergosterol. On the other hand the activation

¹²⁷ E. P. Haussler and E. Brauchli *Helv. Chim. Acta* 12, 187 (1929). I. M. Heilbron and F. S. Spring *Biochem. J.* 24, 133 (1929).

¹²⁸ R. K. Callow *Biochem. J.* 25, 87 (1931).

¹²⁹ A. von Christians and V. Anger *Ber.* 72, 1174, 1482 (1939).

¹³⁰ L. Tschugajeff *Chem. Ztg.* 24, 542 (1900). *Z. anorg. Chem.* 13, 618 (1900).

¹³¹ S. Kon, F. Daniels and H. Steenbock *J. Am. Chem. Soc.* 50, 2573 (1928). A. L. Marshall and A. Knudson *Ibid.* 52, 2304 (1930). T. A. Webster and R. B. Bourdillon *Biochem. J.* 22, 1273 (1928).

R. W. Haman and H. Steenbock *Ind. Eng. Chem. Anal. Ed.* 8, 291 (1936).

¹³² R. S. Harris, J. W. M. Bunker and L. M. Mosher *J. Am. Chem. Soc.* 60, 2579 (1938).

of 7 dehydro cholesterol has been reported¹³³ to be significantly greater by monochromatic light of 296.7 m μ than by any other wave length. Conflicting results have been obtained from studies on the production of vitamin D in rats. While in one laboratory light of 281 m μ was found most effective¹³⁴ in another laboratory a superior effectiveness of light of 296.7 m μ was found¹³⁵.

There are different types of ultraviolet light sources used for the activation of provitamins D namely the light of the magnesium arc and of the carbon arc and of the mercury vapor lamp. The light emitted by a bismuth vapor lamp has also been recommended¹³⁶. Cored carbon electrodes impregnated with various metals are also used.

Provitamins D can be activated in the dry state¹³⁷ in vapor form¹³⁸ and in solutions. Irradiation of provitamin D in the dry state gives poor yields because the vitamin D is produced only on the surfaces of the crystals and further irradiation destroys the vitamin D formed before the provitamin D present in the middle of the crystals has been affected. Irradiation in the vapor phase has not been investigated thoroughly. The best method of irradiation is to expose solutions of provitamins D to the action of the ultraviolet light. Agitation of the solution¹³⁹ was found to enhance the vitamin D yield. The best technical method known today involves the utilization of a special quartz irradiation chamber which is built concentrically around the mercury vapor lamp and through which the provitamin D solution passes continuously in turbulent flow.¹⁴⁰

As pointed out before the yield of vitamin D is influenced by the wave length of the light employed. It is well established that light of wave length between 275 and 300 m μ produces the best yields of vitamin D¹⁴¹ with the smallest amount of by products. The desired light is obtained by special light filters. Light below 275 m μ is filtered out by aromatic compounds such as benzene¹⁴² or xylene¹⁴² or by compounds such as diphenyl¹⁴³ in benzene solution, or by a 5% lead acetate solution.¹⁴⁴ Car

¹³³ J. W. M. Bunker, R. S. Harris and L. M. Mosher, *J. Am. Chem. Soc.* 62, 508 (1940).

¹³⁴ A. Knudson and F. Benford, *J. Biol. Chem.* 124, 287 (1938).

¹³⁵ J. W. M. Bunker, R. S. Harris and L. M. Mosher, *J. Am. Chem. Soc.* 62, 503 (1940).

¹³⁶ N. V. Philips Gloeilampenfabrieken, Holland, Dutch P. 35,479, U. S. P. 1,904,751.

¹³⁷ H. H. Beard, R. E. Burk, H. E. Thompson and H. Goldblatt, *J. Biol. Chem.* 96, 307 (1932).

¹³⁸ P. A. Askew, R. B. Bourdillon and T. A. Webster, *Biochem. J.* 26, 814 (1932).

¹³⁹ A. Wodaus, K. Westphal, F. v. Werd and O. Rygh, *Nachr. Ges. Wiss. Göttingen Math. physik. Klasse* III, 45 (1939).

¹⁴⁰ F. Seitz, *Die Stellung von Vitamin D in der Ernährung*, Leipzig, 1939, 50.

¹⁴¹ E. H. Reckink and A. van Wijk, *Strahlentherapie* 40, 728 (1931). T. H. Rider, G. Sperti, G. P. Goode and H. G. Casady, *J. Am. Med. Assoc.* 106, 452 (1936).

¹⁴² N. V. Philips Gloeilampenfabrieken, G. P. 634,145.

¹⁴³ I. O. Farbenindustrie, G. P. 365,900.

¹⁴⁴ General Development Lab., Inc., U. S. P. 1,982,079.

bon tetrachloride is used to filter out light of the wave lengths 312 and 313 $m\mu$ ¹⁴⁵ Special types of glass which selectively allow light of 270-300 $m\mu$ to penetrate can also be made

The presence of oxygen during the irradiation should be carefully avoided¹⁴⁵ The intermediates and by products formed during the irradiation are much more susceptible to oxidation by molecular oxygen than either the provitamins or the vitamins D The presence of oxygenated materials makes it difficult to isolate crystalline vitamin D, but the actual yield is not essentially affected¹⁴⁷

There is apparently also a specific solvent effect involved in the irradiation procedure The activation takes place more rapidly in ether than in alcohol¹⁴⁸

Other materials such as cyclohexane and the diether, dioxane have also been recommended¹⁴⁹ as solvents either alone or in mixture with ethyl acetate benzene or triethanolamine Provitamins D can also be irradiated in oil solution Of further interest are some special methods of irradiation Thus it is claimed that enhanced yields of vitamin D are obtained when compounds are added which protect the vitamin D after its formation Ethylene or alkalis have been used in this manner¹⁵⁰ The irradiation can also be carried out in the presence of photosensitizers, for example of eosin erythrosin or dibromo dinitro fluorescein¹⁵¹

The temperature coefficient of activation, if it exists, is very small¹⁵² The enhanced effect observed when irradiating at the boiling point of the solvent¹⁵³ is probably due to more uniform activation of all molecules present

The best yields are obtained when only 40 to 60% of the total provitamin D is converted¹⁵⁴ In such cases, the yields are between 30% and 60% of theory This calculation includes the recovery of unchanged provitamin D

¹⁴⁵ N V Philips Gloeilampenfabrieken Holland B P 385 626

¹⁴⁶ A Smakula *Nachr Ges Wiss Göttingen Math physik Klasse* III 49 (1928) C E Bills E M Honeywell and W M Cox *J Biol Chem* 80 557 (1928) E H Reerink and A van Wijk *Biochem J* 23 1994 (1929)

¹⁴⁷ T C Angus F A Askew R B Bourdillon H M Bruce R K Callow C Fischermann J S L Philpot and T A Webster *Proc Roy Soc (London)* B108 340 (1931)

¹⁴⁸ C E Bill E M Honeywell and W M Cox *J Biol Chem* 92 601 (1931)

¹⁴⁹ Standard Brands Inc U S P 1 955 504

¹⁵⁰ I G Farbenindustrie B P 321 992

¹⁵¹ E Merck Co B P 286 664

¹⁵² C E Bills and F G Brickwedde *Nature* 121 452 (1928) T A Webster and R B Bourdillon *Biochem J* 22 1223 (1928)

¹⁵³ I G Farbenindustrie U S P 1 896 191 Société Usines Chimiques du Rhône Poulenc B P 335 277

¹⁵⁴ N V Philips Gloeilampenfabrieken G P 634 146

Direct irradiation of foods has attracted special attention. Thus, cereals and flour have been commercially irradiated. Of considerable practical importance is the irradiation of milk which is carried out in special equipment since ultraviolet light penetrates milk only slightly and since the odor of milk is easily influenced by ultraviolet light. Yeast and dried milk are also irradiated commercially.

(b) Activation by Other Means

Although activation by ultraviolet light has been the only method thoroughly investigated a number of other methods are known for the conversion of provitamins D into vitamins D.

Cathode rays as such¹⁵⁵ or in the presence of catalysts¹⁵⁶ such as iron-uranium salts canal rays¹⁵⁷ α , β and γ rays of radioactive elements,¹⁵⁷ radium emanation¹⁵⁸ x rays^{157, 19} corpuscular rays electrons of high frequency¹⁶⁰ and finally alternating current of high frequency¹⁶¹ have been claimed to effect activation of provitamins D. Most of these claims must be further investigated before they can be accepted. The conversion of provitamins to vitamins D by mitogenetic radiation¹⁶² has also been postulated and will be discussed in the section on the Biogenesis of Vitamin D (page 404).

12 Mechanism of Activation

The conversion of provitamins D to vitamins D is not a simple process. During the course of this reaction several substances are formed^{163, 164, 165} before the vitamin D is obtained and the latter is not stable to the activating energy but is transformed into other compounds.

The mechanism of the provitamin D conversion to vitamin D has been studied extensively in the case of ergosterol. The activating energy was

¹⁵⁵ A. Knudson and C. N. Moore, *J. Biol. Chem.* 81, 49 (1929). R. M. Hoffman and F. Dan, *ibid.* 115, 119 (1936).

¹⁵⁶ American Research Prod. Inc. U. S. P. 1,983,944.

¹⁵⁷ K. Hembold and Vitam. F. Brink, *G. P.* 577,170.

¹⁵⁸ R. B. Moore and T. DeVries, *J. Am. Chem. Soc.* 53, 9676 (1931).

¹⁵⁹ See however H. Goldblatt, *E. geb. Allg. Path. and Path. Anal.* 2, Abt. 25, 58 (1931).

¹⁶⁰ Brit. Thomson Houston Co. B. P. 292,976.

¹⁶¹ I. G. Farb. industries, Austrian P. 119,210.

¹⁶² H. M. 1, *Abhandl. aus der K. k. u. k. schen Grenzgeb.* 1937, H. 45.

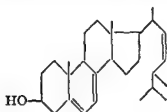
¹⁶³ C. E. Bills and F. G. Brickwood, *Nat. v.* 121, 452 (1928). T. A. Webster and R. B. Bourdillon, *Biochem. J.* 22, 1223 (1928).

¹⁶⁴ A. Smakula, *Nach. Ges. Wiss. Göttingen Math. physik. Klasse* III, 49 (1928). C. E. Bills, E. M. Honeywell and W. M. Cox, *J. Biol. Chem.* 80, 557 (1928). E. H. Reerink and A. van Wijk, *Biochem. J.* 23, 1794 (1929).

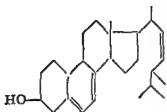
¹⁶⁵ O. Rosenheim and T. A. Webster, *Lancet* II, 629 (1927).

supplied in these studies by ultraviolet light. It is not certain that the same intermediate products, vitamin D and final products are obtained by processes other than the ultraviolet irradiation. It seems relatively certain, however, that at least the vitamin is identical from all forms of activation.¹⁶⁶ The mechanism, which will be described for ergosterol in the following paragraphs, is believed to be identical for all provitamins D and certain indications are available for this assumption other than analogy, especially in the case of the activation of 7 dehydro cholesterol¹⁶⁷ and 22 dihydro ergosterol.¹⁶⁸

The photochemical process is irreversible, that is, there is no equilibrium between the irradiation products. The number of the irradiation products and the sequence of these compounds during the course of the process were revealed by actual isolation of the pure intermediates and by a determination of all the products obtained from each isolated intermediate upon further irradiation. The result of these investigations as pictured in the following scheme for the reaction mechanism.¹⁶⁹



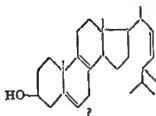
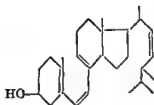
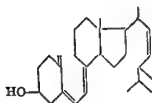
Ergosterol, m p 166° C
 $[\alpha]_D^{20} = -132$ in chloroform
 Absorption maxima at 260 270
 282 and 293.5 μ



Lumisterol, m p 118° C
 $[\alpha]_D^{19} = +192^\circ$ in acetone
 Absorption maxima at 265 and
 280 μ



¹⁶⁶ For the vitamin made by activation of ergosterol by low velocity electrons see I. McQuarrie, W. H. Thompson, A. V. Stoesser and L. G. Rigler *J. Pediatr.* 10: 295 (1937).
¹⁶⁷ A. Windaus, M. Deppe and W. Wunderlich *Ann.* 533: 118 (1937).
¹⁶⁸ A. Windaus and B. Guntzel *Ibid.* 538: 120 (1939).
¹⁶⁹ F. Setz *Z. physiol. Chem.* 215: 183 (1933).

Pro-tachysterol₁Tachysterol₂ $[\alpha]_D^{18} = -70$
in benzene Absorption maxima
at 268 280 and 294 $m\mu$ Vitamin D₂ (Calciferol) M p
116° C $[\alpha]_D^{20} = +106^\circ$ in
alcohol Absorption maximum
at 265 $m\mu$ Toxisterol₂
Absorption maxi-
mum at 248 $m\mu$ Suprasterol₂ I
M p 101° C $[\alpha]_D^{25} =$
-76 in chloroform Ab-
sorption only in far ultra-
violetSuprasterol₂ II
M p 110° C $[\alpha]_D^{25} = +63$
in chloroform Absorption only
in far ultraviolet

While there is no assurance that all intermediate products of the irradiation of ergosterol have been recognized, there is no evidence that other products are formed. During irradiation, the ultraviolet absorption characteristics change with the compounds obtained. This change is illustrated by the following curves of the irradiation products (Fig 16).

All reaction products of the above scheme are isomers of the provitamins

The constitutions of lumisterol, tachysterol and vitamin D are well established, the formula for pro tachysterol is hypothetical

The nomenclature of the irradiation products of the various provitamins D has been proposed by Windaus¹⁷⁰. Accordingly, the intermediates will be called lumisterol, 'pro tachysterol' "tachysterol" "vitamin D, etc, and the special structure due to their derivation from specific provitamins D will be designated by small index numbers such as vitamin D₁ vitamin D₂ lumisterol₁," etc. The index numbers for all irra

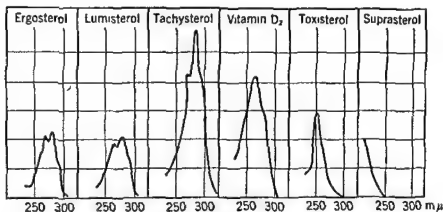


Fig 16—Absorption curves of ergosterol and its irradiation products in 0.2% etheral solution (A Windaus)

diation products from one provitamin are the same, for example all products derived from ergosterol are called products₂, from 7 dehydro cholesterol, products₃, from 22 dihydro ergosterol, products₄. There are no products₁. The term vitamin D₁ was a misnomer given originally to the first isolated crystalline material of vitamin D activity¹⁷¹ which later proved to be a molecular addition product of lumisterol₂ and vitamin D₂¹⁷².

All photochemical reaction products formed upon irradiation of any given provitamin D are subject to further photochemical attack. The irradiation can however, be directed so that one or the other product is obtained predominantly according to the wave length used for the reaction.

Of all the irradiation products formed and isolated only the one which is called 'vitamin D' exerts antirachitic action. The others are physiologically inert or exhibit physiological properties entirely different from those of the natural vitamins D.

¹⁷⁰ A. Windaus, M. Deppe and W. Wunderlich, *Ann.* 533, 118 (1937).

¹⁷¹ A. Windaus, *Proc. Roy. Soc. (London)* B108, 568 (1931). A. Windaus, A. Lüttringhaus and M. Deppe, *Ann.* 489, 252 (1931).

¹⁷² A. Windaus and A. Lüttringhaus, *Z. physiol. Chem.* 203, 70 (1931). A. Windaus, O. Linsert, A. Lüttringhaus and G. Weidlich, *Ann.* 492, 296 (1932).

13 Chemistry of Activation Products

(a) **Lumisterol (Sterol X)** The first irradiation product of ergosterol which can be detected is lumisterol₂. Lumisterol₂ can be prepared predominantly by irradiation of ergosterol with light of the wave length 290–300 mμ¹⁷³. Upon conversion of about 40% of the ergosterol used the unchanged ergosterol is separated by crystallization from methanol. The mother liquors are evaporated to dryness and upon crystallization from acetone the molecular addition product of vitamin D₂ and lumisterol₂ which was called 'vitamin D₁' separates. The addition product is broken up by acetylation, followed by fractional crystallization from acetic acid, whereby the lumisterol₂ acetate crystallizes first. Lumisterol₂ itself is obtained by saponification of its acetate.

Experimental investigations¹⁷⁴ have been made to ascertain if lumisterol₂ is a necessary intermediate or if ergosterol can be converted directly into the next intermediate tachysterol₂. From the changes involved in the absorption spectrum of ergosterol and of lumisterol₂ upon short irradiation with ultraviolet light it is concluded¹⁷⁵ that lumisterol₂ must be formed before tachysterol₂ can be obtained.

Analysis and molecular weight determinations indicate that lumisterol₂ is an isomer of ergosterol. The hydroxyl is present since esters can be formed. Lumisterol₂ contains three double bonds as evident from titrations with perbenzoic acid and from catalytic hydrogenation^{176, 177}. One of these is in the side chain in 22, 23 position as in ergosterol since upon ozonolysis methyl isopropyl acetaldehyde is obtained¹⁷⁸. The other two double linkages are conjugated as evident from the absorption spectrum (Fig. 17). Since upon total dehydrogenation of lumisterol₂ with selenium the same hydrocarbon C₁₈H₁₈ (γ methyl cyclopenteno phenanthrene) is obtained as from ergosterol¹⁷⁹ and since upon nitric acid oxidation of lumisterol₂ and of ergosterol the same toluene tetracarboxylic acid is obtained¹⁸⁰ (see page 356) it must be concluded that the original ring system of ergosterol is also the ring system of lumisterol₂ and that the system of conjugated double bonds is present in one ring which can be only ring B or ring C of the sterol ring skeleton. Lumisterol₂ upon treat

¹⁷³ A. Windaus, K. Dithmar and E. Fernholz, *A*, 493, 265 (1933).

¹⁷⁴ H. Lettré, *Ibid.*, 511, 280 (1934).

¹⁷⁵ K. Dimroth, *B*, 70, 1631 (1937).

¹⁷⁶ K. Dimroth, *Ibid.*, 68, 33 (1933).

¹⁷⁷ A. Windaus, K. Dithmar and E. Fernholz, *Ann.*, 493, 265 (1933).

¹⁷⁸ A. Guteras, Z. Nakmoya and H. H. Imhoff, *Ibid.*, 494, 116 (1932).

¹⁷⁹ H. H. Imhoff, *Ibid.*, 494, 172 (1932).

¹⁸⁰ G. Ahrens, E. Fernholz and W. Stoll, *Ibid.*, 500, 109 (1933).

ment with perbenzoic acid yields a triol, which gives only a diacetate, and upon treatment with mercuric acetate yields dehydro lumisterol.¹⁸¹ All these reactions are in strict analogy to the behavior of ergosterol so that there is little doubt that the double bonds are in the same position as they are in ergosterol, namely, in the 5,6 and in 7,8 positions. On the other hand, lumisterol₂ does not form an addition product with digitonin and does not form a bimolecular compound upon irradiation in the presence of eosin. Furthermore, upon total hydrogenation a hexahydro compound is formed which is different from that obtained from ergosterol. The difference cannot be due to isomerization of the 3 hydroxyl group

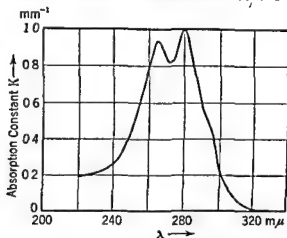


Fig 17 —Absorption spectrum of lumisterol₂
(H Brockmann)

since pyro calciferol which will be discussed later, also yields a bimolecular compound upon irradiation with eosin. It must therefore be concluded that the difference between ergosterol and lumisterol is only in the steric position of one substituent on one of the asymmetrical carbon atoms 9 or 10. Actually an isomerization on carbon atom 10 is involved as will be discussed in the section on the pyro calciferols (page 399).

Lumisterol can be converted into vitamin D₂ by irradiation but is itself devoid of antirachitic efficacy.

Lumisterol₃¹⁸² and lumisterol₄¹⁸³ have been isolated from the irradiation products of 7 dehydro cholesterol and 22 dihydro ergosterol. It is interesting to note that these lumisterols in contradistinction to lumisterol₂ do not form molecular addition compounds with their corresponding vitamin D.

¹⁸¹ I. M. Heilbron, F. S. Spring and P. A. Stewart, *J. Chem. Soc.* 1935 1221. I. M. Heilbron and F. S. Spring, *Chem. and Ind.* 54 795 (1935).

¹⁸² A. Windaus, M. Deppe and W. Wunderlich, *Ann.* 533 118 (1937).

¹⁸³ A. Windaus and B. Guntzel, *Ibid.* 538 170 (1939).

The formation of lumisterol compounds from three different provitamins D by ultraviolet irradiation allows the prediction that similar lumisterol compounds are formed by all steroids which have the system of conjugated double bonds and the steric configuration of the three provitamins ergosterol 7 dehydro cholesterol and 22 dihydro ergosterol. The changes in the absorption spectrum of $\Delta^{5,7}$ androstadiene diol 3,17 upon irradiation make the existence of a lumisterol compound certain. That the exact position of the double bonds in the starting material is necessary for the lumisterol formation is evident from the fact that isodehydro cholesterol upon irradiation does not form a corresponding lumisterol compound. The steric specificity for the lumisterol formation is apparent since pyro calciferol and isopyro calciferol (see page 399) do not yield lumisterol derivatives.

(b) **Pro-tachysterol** Pro tachysterol is obtained from lumisterol upon irradiation. It has not been isolated in the pure form and is apparently not stable, but undergoes rearrangement into tachysterol as evident from spectroscopical studies¹⁸⁴. Thus crude irradiation products of ergosterol kept sealed in the absence of oxygen change their absorption spectrum. While this dark reaction is slow at room temperature it can be brought about in a few hours by heating to 55° C. The spectral changes involve the appearance of the typical absorption spectrum of tachysterol at 280 m μ .

(c) **Tachysterol**¹⁸⁵ Tachysterol follows pro tachysterol and precedes the vitamin in the sequence of irradiation products.

Tachysterol₂ can be obtained¹⁸⁶ from ergosterol by irradiation with the shorter wave lengths of the ultraviolet light until about 60% of the ergosterol is transformed. The separation from unchanged ergosterol is effected by crystallization from methanol. Tachysterol₂ is isolated from the other irradiation products by means of citraconic anhydride which forms an addition compound with tachysterol₂. Tachysterol₂ itself is obtained from the adduct by thermal decomposition. While tachysterol₂ could not be obtained in crystalline form the 3,5 dinitro-4 methyl benzoic acid ester forms well shaped crystals.

The constitution of tachysterol appears to be definitely established. The outstanding property of tachysterol is the ease with which it is autooxidized. This is considerably greater than the oxidation of ergosterol or of any of the other irradiation products. Analysis of the crystallized ester established that tachysterol₂ is an isomer of ergosterol. Tachysterol₂

¹⁸⁴ A Windaus and L. Auhgen, *Z. phys. Chem.* 195, 103 (1931).

¹⁸⁵ The name tachysterol has been given to this compound in recognition of its outstanding property namely the speed with which it reacts (ox. Gr. = fast).

¹⁸⁶ A Windaus, F. v. Werder and A. Löttringhaus, *Ann.* 499, 183 (1933).

contains four double bonds that is one double bond more than ergosterol or lumisterol.¹⁸⁷ This was established by the following series of reactions. Tachysterol acetate forms an addition product with citraconic anhydride as mentioned before. The formation of this adduct requires that one double linkage disappears. Catalytic hydrogenation of the adduct re-

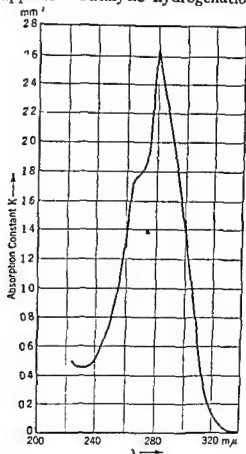


Fig 18 — Absorption spectrum of tachysterol₂ (H Brockmann)

vealed that two more double bonds are present. The tetrahydro tachysterol₂ acetate citraconic anhydride is however not a saturated compound since the presence of one more double bond can be demonstrated upon titration with perbenzoic acid. The presence of four double linkages requires the presence of only three rings instead of the four rings present in ergosterol and in lumisterol. The fourth additional double bond which is formed by ring opening must be in conjugation to the two double bonds in ring B as evident from the absorption spectrum which shows maxima at 268 280 and 294 m μ (Fig 18).

¹⁸⁷ H. I. Jettre, *Ann.* 511 280 (1934)

The ring opening during the formation of tachysterol₂ from lumisterol₂ occurs between carbon atoms 9 and 10. This has been assumed in view of the fact that in vitamin D₂ which follows tachysterol₂ in the sequence of irradiation products ring B is open between carbon atoms 9 and 10 as has been proved by oxidation experiments (see page 392). The close relationship of tachysterol₂ to vitamin D₂ has also been demonstrated by hydrogenation with sodium in alcohol both compounds yielding the identical dihydro derivative¹⁸⁸ (dihydro vitamin D₂I).

The position of the three conjugated double bonds has been established by oxidation experiments¹⁸⁹. Vitamin D₂ yields upon oxidation a ketone C₁₉H₃₂O which will be discussed in the section on the Constitution of the Vitamin (see page 396). This ketone is obtained upon cleavage of the double bond between carbon atoms 7 and 8. Since the same ketone could not be obtained under similar oxidation conditions from tachysterol₂ it must be concluded that the latter has no double bond in the 7,8 position. The positions of the three conjugated double bonds must therefore be assumed to be in the 10,5, 6,7 and 8,9 positions.

Tachysterol₂ has no antirachitic action and was found to be about half as toxic as vitamin D₂. Tachysterol₃ is formed from 7 dehydro cholesterol¹⁹⁰ and tachysterol₄ from 22 dihydro ergosterol¹⁹¹ in a manner similar to the formation of tachysterol₂ from ergosterol. The isolation of these compounds is carried out by means of the condensation compounds with citraconic anhydride as described. None of the known tachysterols is a crystallized compound but crystalline esters have been obtained.

Dihydro tachysterol₂, AT 10 (*Anti tetany compound No 10*). Dihydro tachysterol₂ is of considerable theoretical and practical importance. It is prepared¹⁹² by sodium and alcohol reduction of the 3,5 dinitro 4 methyl benzoic acid ester of tachysterol followed by saponification (Dihydro vitamin D₂I is obtained in about 30% yield as a by product in the reaction). While tachysterol₂ has not been obtained in the pure crystalline form the dihydro derivative₂ can easily be obtained in such a state.

Dihydro tachysterol₂ is slightly active antirachitically¹⁹³. It causes an increase of the calcium concentration in the blood. Tachysterol itself

¹⁸⁸ M. Müller *Z. phys. Chem.* 233 2 3 (1935)

¹⁸⁹ W. Grundmann *Ibid.* 252 151 (1936)

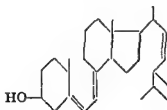
¹⁹⁰ A. Wadus, M. Depp and W. Wundelich *Ann.* 533 118 (1937)

¹⁹¹ A. Wadus and B. G. Atel *Ibid.* 538 10 (1937)

¹⁹² I. G. Farbenindustrie U. S. P. 2,001,117

¹⁹³ F. W. W. *Z. physiol. Chem.* 260 119 (1939)

has the same property but is only $1/10$ as active. The dihydro compound is used clinically under the name 'A T 10' for the treatment of idiopathic and postoperative (hypoparathyroid) tetany.^{194 195 196} Dihydro tachysterol has the following formula, as is evident from absorption spectrum, catalytic hydrogenation and oxidative degradation reaction.¹⁹⁷



M p 125-127° C
Absorption maxima at 242
251 and 261 mμ

(d) **Vitamin D** The vitamins D follow the tachysterols in the sequence of photochemical reaction products from provitamins D. The isolation properties and chemical constitution of the vitamins D will be described later.

The vitamins D are the only photoisomers of the provitamins D with antirachitic efficacy.

(e) **Toxisterol (Substance 248)** Upon further irradiation of vitamin D a toxic compound is formed which has not yet been isolated in the pure state. It is characterized by its absorption spectrum which shows a maximum at 248 mμ. It has no antirachitic activity¹⁹⁸ but is quite toxic.^{199 200} There is considerable evidence that this compound is formed more readily when the irradiation is carried out in alcohol than in ether.²⁰¹

¹⁹⁴ F. Holtz *Merck's Jahresber.* 47, 0 (1934).

¹⁹⁵ F. Holtz *Klin. Wochschr.* 13, 104 (1934); *Deut. med. Wochschr.* I, 560 (1931); II, 1830 (1934).

¹⁹⁶ M. MacBryde *J. Am. Med. Assoc.* 111, 304 (1938); J. A. Greene and L. W. Swanson *J. Iowa Med. Soc.* 29, 275 (1939); O. C. Pickhardt and A. Bernhard *Ann. Surg.* 108, 362 (1938); E. Rose and F. W. Sunderman *Arch. Intern. Med.* 64, 217 (1939); L. M. Hurxthal and T. S. Claiborne *New England J. Med.* 220, 911 (1939).

¹⁹⁷ F. v. Werder *Z. physiol. Chem.* 260, 119 (1939).

¹⁹⁸ A. Smakula *Nachr. Ges. Wiss. Göttingen Math. physik. Klasse* III, 49 (1938); C. E. Bills, E. M. Honeywell and W. M. Cox *J. Biol. Chem.* 80, 557 (1928); E. H. Reerink and A. van Wijk *Biochem. J.* 23, 194 (1929).

¹⁹⁹ A. Windaus, A. Luttringhaus and P. Busse *Nachr. Ges. Wiss. Göttingen Math. physik. Klasse* III, 150 (1932).

²⁰⁰ F. Laquer and O. Linsert *Klin. Wochschr.* 12, 723 (1933).

²⁰¹ A. van Wijk and E. H. Reerink *Nature* 122, 648 (1928); W. E. Dixon and J. C. Hoyle *Brit. Med. J.* II, 832 (1928); L. J. Harris and T. Moore *Biochem. J.* 23, 261 (1929); J. C. Hoyle and H. Buckland *Ibid.* 23, 558 (1929); J. C. Hoyle *J. Pharm.* 40, 351 (1930); R. Kern, M. F. Montgomery and F. W. Still *J. Biol. Chem.* 93, 365 (1931).

The compound is called toxisterol or substance 248 because of its outstanding ultraviolet absorption at 248 $m\mu$ (Fig 19)

(f) Suprasterols I and II The information available concerning the formation of the irradiation products of vitamin D is scarce It seems²⁰² that toxisterol and the two suprasterols I and II are formed simultaneously, but it has also been assumed²⁰³ that toxisterol precedes the suprasterols

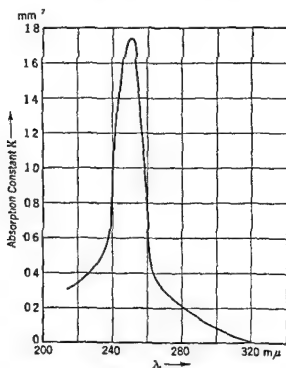


Fig 19—Absorption spectrum of toxisterol;
(H Brockmann)

The two suprasterols appear to be the photochemical end products from the irradiation of the provitamins

The suprasterols, I and II have been obtained²⁰⁴ from over irradiated ergosterol They have been isolated by fractional crystallization of their allophanates whereby the suprasterol, I is obtained first The suprasterols are isomers of the provitamins from which they have been obtained In the case of the suprasterols, I and II the presence of the hydroxyl group and the side chain as present in ergosterol have been proved

²⁰² P Setz *J physiol Chem* 215 183 (1933)

²⁰³ C E Bills *Physiol Rev* 15 1 (1935)

²⁰⁴ A Windaus *J Gaede J Köser and G Stein Ann* 483 17 (1930)

Suprasterol₁ I has three double bonds according to the results obtained from catalytic hydrogenation and from titration with perbenzoic acid.²⁰⁵ Since neither of the suprasterols shows any absorption in the ultraviolet region above 240 m μ (Fig. 20), it must be concluded that the double bonds are not conjugated. The presence of only three double bonds suggests that ring closure occurred upon irradiation of vitamin D. The typical

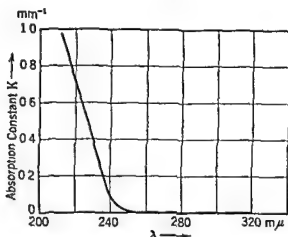
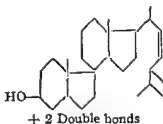


Fig. 20—Absorption spectrum of the suprasterols, (H. Brockmann)

hydrocarbon, γ methyl cyclopenteno phenanthrene, which is obtained from all sterol compounds upon dehydrogenation with selenium, was not obtained from either of the suprasterols. The ring closure has therefore not resulted in the formation of the sterol skeleton.

Even less is known about suprasterol₂ II. There appear to be three double bonds, but there is no conclusive evidence to substantiate this.

As long as the structure is not certain, no definite formulas can be given for the suprasterols. The following spiro cyclopentane formula has been tentatively suggested.²⁰⁶



²⁰⁵ M. Müller *Z. physiol. Chem.* 233 223 (1935)

²⁰⁶ M. Müller *Ibid.* 233 223 (1935)

By over irradiation of 7 dehydro cholesterol³⁰⁷ and of 22 dihydro ergosterol³⁰⁸ the suprasterols₃ and 4 respectively are formed

VITAMINS D

The number of naturally occurring vitamins D is unknown and it is an extremely difficult task to isolate and to identify the pure vitamin D from any source. Prior to the isolation of any pure antirachitic substance from natural material, various forms of vitamin D were obtained by irradiation of different provitamins. Numbers were assigned to these various vitamins D in order to simplify the nomenclature. Thus today we have the following series of five vitamins D with their corresponding provitamins

TABLE IV

Vitamin D	Provitamin D	Remarks
Vitamin D ₁	Ergosterol	Vitamin D ₁ is a molecular compound consisting of vitamin D ₂ and lumisterol ₂
Vitamin D ₂	Ergosterol	Vitamin D ₂ is also called calciferol or viosterol
Vitamin D ₃	7 Dehydro cholesterol	Vitamin D ₃ is also referred to as dimethyl dihydro calciferol
Vitamin D ₄	22 Dihydro-ergosterol	
Vitamin D ₅	7 Dehydro sitosterol	

Actually, since at least ten different provitamins D are known ten different vitamins D also should be listed. But only the four vitamins, designated vitamins D₂₋₅ have been prepared in essentially pure form. Whether or not the four vitamins D listed above occur in nature is not known. Only vitamin D₂ and vitamin D₃³⁰⁹ have been isolated in the pure form from fish liver oils. Furthermore another vitamin D of unknown constitution has been separated by molecular distillation from fish liver oils. From the properties of this vitamin it is inferred that it is not identical with any of the above listed vitamins and it will therefore be referred to in this monograph provisionally as vitamin D₆₍₁₎. Besides vitamin D₆₍₁₎ five other vitamins D occur in cod liver oil as evident from

³⁰⁷ A. W. and U. M. D. Ipe and W. Wundrich *Ann.* 533 119 (1937)

³⁰⁸ A. W. and U. M. D. Ipe and W. Wundrich *Ann.* 538 10 (1939)

³⁰⁹ H. Brockmann and A. Buise *Z. physiol. Chem.* 256 37 (1938)

molecular distillation experiments. Of these six vitamins D, two are present as the major constituents, two are present in lesser quantities and the last two are present only in traces.²¹⁰ The distillation curves for the vitamins D from spearfish and from 'white sea bass' are different from each other and from those of cod liver oil. This suggests that these fish contain different vitamins D.

The complexity of the vitamins D which occur in nature can also be demonstrated by biological experiments, using different species of animals as test objects. All vitamins D are primarily standardized on rats. However, when tested on chicks, the naturally occurring vitamins D show quite inconsistent responses, Rat Unit for Rat Unit. In order to have a standardized vitamin D with which other vitamin D preparations can be compared, a special preparation of cod liver oil has been selected in the United States as a reference. This material is carefully tested on rats and the response of this preparation on chicks is designated arbitrarily as a 100% activity, that is, one Rat Unit vitamin D from the U. S. Reference Cod Liver Oil is arbitrarily taken as one Chick Unit of vitamin D. Vitamin D₃ also proved to be 100% chick active. On the other hand vitamin D₂ has only 1/2 to 1/4 the chicken activity of the U. S. Reference Cod Liver Oil. Vitamin D₂ has practically no chicken activity. The livers of some fish contain vitamins D which are much more than 100% chick active. Thus vitamin D from the 'white sea bass' (*Cynoscion nobilis*) has been reported to be over 300% chick active and the vitamin D from the dogfish (*Squalus suckleyi*) about 230% active.²¹¹ Careful analysis of these results lead the investigators to the belief that these high chicken potencies of special fish oils are due to a real vitamin D, that is, due to at least one new, so far unknown form, and that these potencies cannot be explained on the basis of some hypothetical synergistic factor.

The species specificity has also been demonstrated with turkeys. A Chick Unit is not necessarily a Turkey Unit of vitamin D.²¹² Further, more some evidence exists that human beings react preferentially to certain forms of naturally occurring vitamin D. Thus it has been observed that seal oil is a much more effective antirachitic agent for humans than for rats.²¹³ There is also ample evidence that vitamin D₃ is somewhat more effective in man than vitamin D₂.

²¹⁰ K. C. D. Hickman and E. L. Gray *Ind. Eng. Chem.* 30, 796 (1938).

²¹¹ C. E. Bills, O. N. Massengale, M. Imboden and H. Hall *J. Nutrition* 13, 435 (1937).

²¹² T. H. Jukes and T. D. Sanford *Ibid.* 18, 71 (1939).

* ²¹³ E. J. Mikhlin, M. J. Leizerovskaya and N. N. Milovanova *Kazan Med. Zhur.* 33, 64 (1937) *Chem. Zentr.* 1938 II, 3108.

14 Occurrence

Vitamin D occurs in nature only in small amounts. The living plant tissue and fresh green vegetables contain no detectable amount of this vitamin. The occurrence of very small amounts of vitamin D in some species however is possible due to the presence of significant amounts of provitamin D. Thus by irradiation from the sunlight a certain amount of vitamin D should be formed.

From time to time it has been reported that certain plant materials contain considerable amounts of vitamin D. It has not been possible however to verify these claims. On the other hand it has been found that yeasts and molds grow readily on many non living plants. These organisms contain significant amounts of provitamins D as discussed previously (page 345). A transition of these provitamins into vitamins D under the influence of sunlight has been proved in many cases, for example in the alleged antirachitic activity of cacao shells²¹⁴ and of hay²¹⁵.

Vitamin D occurs only in small quantities in most members of the animal kingdom.¹⁶ Only a few classes of animals and of animal products contain significant amounts.

Abundant quantities of vitamin D are present in the livers and also to a certain extent in the viscera of fish. The actual amount of vitamin D per gram of liver oil varies considerably with the species, the season and a number of other biological factors such as age, climate, food supply, condition of living, etc. Thus for example halibut livers give in the summer months a high yield in oil of low potency while in the winter months less oil with more vitamin D is obtained. As a general rule it appears that fish with much body oil are the richest natural source of vitamin D. The distribution of vitamin D in various fish oils may be illustrated by Table V.

While the fat from fish contains relatively large amounts of vitamin D, the fat of other animals contains little or none.²¹⁶ Exceptions to this general rule are certain fats from animals, especially from certain birds that live upon fish. Furthermore the nutritive animal material for the initial growth of those species that require vitamin D contains small but significant amounts. Thus the eggs of birds are a relatively good source. More specifically the yolks of hen and duck eggs contain definite amounts which are usually fairly constant (see page 418). The milk of mammals contains vitamin D. Furthermore inasmuch as milk contains the opti

²¹⁴ A. W. Knapp and K. H. Coward *Analyt* 59 474 (1934)

²¹⁵ H. Steenbock, I. B. Hart, C. A. Elvehjem and S. W. F. Kletz *J. Biol. Chem.* 66 499 (1925)

²¹⁶ P. A. Coppens and G. A. Metz *Arch. n. erl. d. physiol.* 18 407 (1933)

TABLE V

DISTRIBUTION OF VITAMIN D IN VARIOUS FISH OILS

Source of oil	Zoological name	Potency I U per G
Bluefin tuna liver	<i>Thunnus thynnus</i>	40 000
Swordfish liver	<i>Xiphias gladius</i>	10 000
Yellowfin tuna liver	<i>Neothunnus macropterus</i>	10 000
Black sea bass liver	<i>Stereolepis gigas</i>	5 000
Bocaccio liver	<i>Sebastes paucispinus</i>	2 100
Red rockfish liver	<i>Sebastes ruberrimus</i>	1 500
Black rockfish liver	<i>Sebastes mystinus</i>	1 500
China rockfish liver	<i>Sebastes nebulosus</i>	1 400
Ling cod (not codfish) liver	<i>Ophiodon elongatus</i>	1 300
Chinook salmon liver	<i>Oncorhynchus tshawytscha</i>	1 300
Halibut liver	<i>Hippoglossus hippoglossus</i>	1 200
Rabbitfish liver	<i>Chilomycterus schoepfi (?)</i>	1 100
Striped rockfish liver	<i>Sebastes elongatus</i>	1 000
Starry flounder liver	<i>Platichthys stellatus</i>	1 000
Boston mackerel liver	<i>Scomber scombrus</i>	750
Black cod (not codfish) liver	<i>Anoplopoma fimbria</i>	600
Pufferfish liver	<i>Sphoeroides maculatus</i>	570
Dog salmon liver	<i>Oncorhynchus keta</i>	400
Black horse mesentery	<i>Cycleptus elongatus</i>	400
Turbot liver	<i>Reinhardtius hippoglossoides</i>	260
Rex sole liver	<i>Errex zachirus</i>	150
California sand dab liver	<i>Orthopsetta sordida</i>	120
Cod liver	<i>Gadus morrhua</i>	100
Herring entire body	<i>Clupea harengus</i>	100
Yellow sole liver	<i>Pseudopleuronectes dignabilis</i>	90
Sardine entire body	<i>Sardinia caerulea</i>	80
Goosefish liver	<i>Lophius piscatorius</i>	70
Pollack liver	<i>Pollachius virens</i>	50
Menhaden entire body	<i>Brevoortia tyrannus</i>	50
Shark liver	(Sp)	50
Salmon trimmings	<i>Oncorhynchus</i> (Sp)	40
Turbot body minus liver	<i>Reinhardtius hippoglossoides</i>	30
Skate liver	<i>Raja binoculata</i>	25
Dogfish (Pacific) liver	<i>Squalus suckleyi</i>	20
Muddy catfish body	<i>Leptops olivaris</i>	20
Ohio perch mesentery	<i>Aplocheilichthys grunniens</i>	11
Buffalo mesentery	<i>Ictiobus cyprinella</i>	10
Haddock liver	<i>Melanogrammus aeglefinus</i>	10
Channel catfish mesentery	<i>Ictalurus punctatus</i>	5
Dogfish (Atlantic) liver	<i>Squalus acanthias</i>	3
Capelin entire body	<i>Mallotus villosus</i>	3
Ratfish liver	<i>Chismaera collieri</i>	2
Gray sole liver	<i>Glyptocephalus cynoglossus</i>	<1
Sturgeon liver	<i>Acipenser rubicundus</i>	Nil

From C E Bills *Physiol Rev* 15, 13 (1935)

much amounts of calcium and phosphorus it is used extensively for supplementing human needs (see page 403) Milk products, especially dried milk and butter also contain the vitamin originally present in milk.

15 Isolation

The isolation of vitamin D from naturally occurring fish liver oils is carried out by first isolating the unsaponifiable fraction. For this purpose either the entire oil or the alcoholic extract therefrom¹⁷ is hydrolyzed. This is done under the most careful conditions, for example by means of potassium hydroxide in methanol in an atmosphere of nitrogen.¹⁸ The next step consists in separating the vitamin D from the vitamin A present. This can be achieved by condensing the vitamin A with maleic anhydride²¹ or, better, with citraconic anhydride.⁹ Another separation method consists in the extraction of the mixture of vitamins A and D in hydrocarbon solution, such as benzene²² or pentane² with aqueous methanol (90–95%) whereby the vitamin D remains in the hydrocarbon, whereas the vitamin A goes into the methanol solution. The best method for further purification is apparently a selective adsorption on aluminum oxide according to the principle of chromatographic adsorption. The position of the vitamin in the adsorption column can be detected by adding to the vitamin solution an indicator, for example indicator red 33,²³ which has the same adsorption characteristics as the vitamin D. The vitamin D is separated from sterols, for example from cholesterol, by freezing in methanol solution²⁴ or by precipitation with digitonin.^{5, 25, 7, 26} Another method for the purification of the vitamin D is its separation from non alcoholic constituents by esterification with phthalic acid anhydride followed by fractionation of the esters.^{29, 20, 21} A certain purification can also be obtained by high vacuum distillation.^{12, 23} Final purification is achieved by esterification, for example with 3,5-dinitro

¹⁷ T. P. Zucker, *Proc. Soc. Exptl. Biol. Med.* 19, 167 (1922); 20, 136 (1922).

¹⁸ O. Neracher and T. Reichstein, *Helv. Chim. Acta* 19, 1382 (1936).

¹⁹ O. Dalmer, F. v. Werdner and T. Moll, *Z. physiol. Chem.* 224, 86 (1934).

²⁰ A. Wandaus, O. Linsert, A. Lüttringhaus and G. Wedlich, *Ann.* 492, 226 (1931).

²¹ H. Brockmann, *Z. physiol. Chem.* 241, 104 (1936); *Ibid.* 245, 96 (1937); H. Brockmann and A. Busse, *Ibid.* 249, 176 (1937).

²² O. Neracher and T. Reichstein, *Helv. Chim. Acta* 19, 1382 (1936).

²³ H. Brockmann, *Z. physiol. Chem.* 241, 104 (1936); *Ibid.* 245, 96 (1937); H. Brockmann and A. Busse, *Ibid.* 249, 176 (1937).

²⁴ O. Neracher and T. Reichstein, *Helv. Chim. Acta* 19, 1382 (1936).

²⁵ H. Brockmann, *Z. physiol. Chem.* 241, 104 (1936); *Ibid.* 245, 96 (1937); H. Brockmann and A. Busse, *Ibid.* 249, 176 (1937).

²⁶ E. J. H. Simons and T. F. Zucker, *J. Am. Chem. Soc.* 58, 2655 (1936).

²⁷ G. A. D. Haslewood and J. C. Drummond, *J. Soc. Chem. Ind.* 55, 598 (1936).

²⁸ O. Neracher and T. Reichstein, *Helv. Chim. Acta* 19, 1387 (1936).

²⁹ F. J. H. Simons and T. F. Zucker, *J. Am. Chem. Soc.* 58, 2655 (1936).

³⁰ F. Ender, *Z. Vitaminforsch.* 2, 241 (1933); 3, 161 (1934).

³¹ O. Neracher and T. Reichstein, *Helv. Chim. Acta* 19, 1382 (1936).

³² F. Ender, *Z. Vitaminforsch.* 2, 241 (1933); 3, 161 (1934).

³³ O. Neracher and T. Reichstein, *Helv. Chim. Acta* 19, 1382 (1936).

benzoyl chloride and purification of the dinitro benzoate by fractional crystallization^{34 235} or by adsorption on aluminum oxide²³⁶ Esterification may also be carried out with isocyanic acid, followed by fractional crystallizations of the allophanates obtained³⁷ The free vitamin D is then obtained by saponification of the esters By utilization of these methods, vitamin D₃ and vitamin D₂ have been obtained²³⁸ from tuna liver oil and from halibut liver oil

The method of separating naturally occurring vitamins D by molecular distillation is also of considerable interest²³⁹ In order to facilitate the distillation process a constant yield oil is added to the fish liver oils (For details of this method see page 61) Upon distillation of such a mixture free and esterified vitamins D can be separated While the former distill around 160° C the latter pass over at 230–250° C The utilization of this method has resulted in the separation of a new vitamin D, which is characterized by the lowest boiling point of the entire vitamin D fraction and which will be provisionally referred to in this monograph as vitamin D₆ (2)

The methods of isolating vitamins D from the irradiation products of the provitamins are somewhat different from the methods used for the isolation of the naturally occurring vitamins D, since a different type of by products must be eliminated The best yield of vitamins D from the irradiation of provitamins D is obtained when about 40 to 60% of the provitamin has been transformed The percentage converted can be determined approximately by crystallization of the unchanged provitamin D from alcohols or somewhat more accurately by precipitation with digitonin Either of these operations, or a combination of both is carried out as the first step in the actual isolation procedure to obtain the pure vitamin D The isolation of the formed vitamin D is carried out in the absence of oxygen Although vitamin D is only moderately sensitive to molecular oxygen at room temperature, the intermediate tachysterol which is always present in the crude irradiation products, at least in small amounts adsorbs oxygen readily with the formation of peroxides which in turn have the tendency to destroy the vitamin D Tachysterol is separated from the irradiation mixture by condensation with citraconic anhydride The crude reaction product is saponified at room temperature

³⁴ H Brockmann and A Busse *Naturwissenschaften* 26 122 (1938)

³⁵ E J H Simons and T F Zucker *J Am Chem Soc* 58 2655 (1936)

³⁶ H Brockmann *Z physiol Chem* 241 104 (1936) *Ibid* 245 96 (1937) H Brockmann and A Busse *Ibid* 249 176 (1937)

³⁷ G A D Ha lewood and J C Drummond *J Soc Chem Ind* 55 708 (1931)

²³⁸ H Brockmann and A Busse *Z physiol Chem* 256 250 (1938)

²³⁹ K C D Hickman *Ind Eng Chem* 29 1107 (1937)

and extracted with petroleum ether and water whereby the tachysterol citraconic acid adduct remains in the water phase while the vitamin D goes into the petroleum ether. The vitamin D is then crystallized from acetone at low temperature and recrystallized from acetone-methanol.

In another method used for the isolation of vitamin D after irradiation of its provitamin a characteristic sparingly soluble ester of the vitamin such as the 3,5-dinitro benzoate²⁴⁰ is prepared. This can be purified by fractional crystallization and the pure vitamin D is obtained by saponification of the ester.

The vitamins D form addition products very easily with other compounds especially with substances that contain hydroxyl groups. This behavior is of course not characteristic for the vitamins D for it is found in the case of many chemicals and especially of sterols. In the case of the vitamins D this problem is, however, of practical importance. Thus the vitamins D crystallize with the solvent of crystallization and especially with water. The molecular compounds which the vitamins D form with other sterols are even more annoying. Thus vitamin D₂ forms an addition product with lumisterol₂ (to give the so called vitamin D₁) and vitamin D₂ forms an addition compound with the irradiation product of iso dehydro cholesterol²⁴¹ which occurs as an impurity in the synthetically obtained 7 dehydro cholesterol.

16 Properties

All the known vitamins D in the pure state, are white odorless crystals. They are soluble in fats and in the usual organic solvents, for example in ether, chloroform, acetone, alcohol and are insoluble in water. They exhibit a characteristic absorption spectrum with one maximum at 265 mμ (in hexane and in ether) the molecular extinction coefficient $\epsilon = 1.82 \times 10^4$ [$k = 45 - 46 \times 10^3$] (Fig. 21).

Vitamin D₂

M p 115-117 C $[\alpha]_D^{20} = +103$ in abs alcohol +82.6 in acetone +33.3 in petroleum ether +91.2° in ether.

3,5-Dinitro benzoate M p 148-149 $[\alpha]_D^{20} = +50$ in benzene.

p Nitro benzoate M p 93 $[\alpha]_D^{20} = +104$ in chloroform.

Stability Crystallized vitamin D₂ sealed in the absence of oxygen and stored in the absence of light at 2 C is stable over a period of many months. When dissolved in olive oil and kept under similar conditions more than half of the original amount is still present after five years. Vitamin D₂ is thermolabile. Although it can be

²⁴⁰ F. A. Askew, R. H. Bourdillon, H. M. Bruce, R. K. Callow, J. S. L. Philpot and T. A. Webster, *Proc. Roy. Soc. (London)* B109 488 (1933).

²⁴¹ H. Brockmann and A. Rine, *Naturwissenschaften* 26 122 (1938).

sublimed at 125° C in high vacuum decomposition occurs at this temperature. The two thermal decomposition products, pyro calciferol and isopyro calciferol, are usually obtained by heating to 160–190° C.

Efficacy 1 g contains 40 million International Units vitamin D

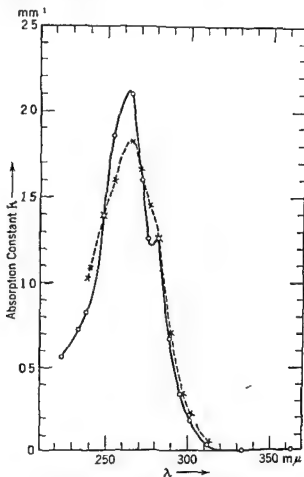


Fig. 21—Absorption spectrum of vitamin D₂ (O—O) and of vitamin D₃ (X—X) in 0.02% solution in hexane (H Brockmann)

Vitamin D₂

M p 82–83° C $[\alpha]_D^{20} = +83.3^\circ$ in acetone

3,5-Dinitro benzoate M p 129 and 140° C (polymorphic) $[\alpha]_D^{20} = +98^\circ$ in chloroform

p-Nitro benzoate M p 127° $[\alpha]_D^{20} = +114^\circ$ in chloroform

Vitamin D₃²⁴²

M p 107–108° $[\alpha]_D^{20} = +89.3^\circ$ in acetone

3,5-Dinitro benzoate M p 135–136° $[\alpha]_D^{20} = +95.4^\circ$ in acetone

²⁴² A. Windaus and C. Trautmann / *physiol. Chem.* 247:185 (1937)

17 Chemical Constitution

The chemical constitution of the vitamins D is closely related to the constitution of the provitamins from which they can be derived. As pointed out on page 350 the provitamins differ from each other only in the number of carbon atoms in the side chain and the degree of unsaturation. The sterol skeleton is the same for all provitamins. This is also true for the vitamins D derived from the provitamins D. All chemically investigated vitamins D have the same constitution with the exception of different structures of the side chain.

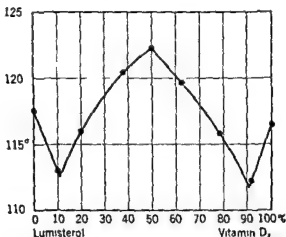


Fig. 22—Melting point diagram of lumisterol and vitamin D₂. (A. Windaus, K. Dithmar and E. Fernholz.)

While the details of the chemical constitution of all the vitamins D have not been investigated, the constitution of vitamin D₂ or calciferol, which is derived from ergosterol, has been totally elucidated. The constitution of the other vitamins D can then be deduced by analogy and in the case of vitamin D₃, which is obtained from 7 dehydro cholesterol, this deduction has been proved to be correct.

(a) *The Constitution of Vitamin D₁*

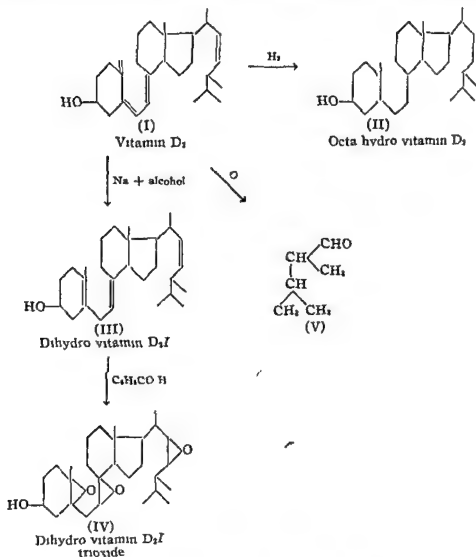
Vitamin D₁ has the constitution of a molecular addition product of vitamin D₂ with lumisterol₂ (see page 375). This has been shown by determination of the melting point diagram (Fig. 22) obtained by mixing various amounts of the two components.

(b) *The Constitution of Vitamin D₂*

Vitamin D₂ has the empirical formula C₂₈H₄₄O²⁴³ It is therefore an isomer of ergosterol, from which it is derived The constitution of vitamin D₂ can consequently be linked with the structure of ergosterol (see page 350)

The oxygen present is in a hydroxyl group in the vitamin D₂ molecule, as is evident from the formation of esters

Vitamin D (I) contains four double bonds since upon catalytic hydrogenation four mols of hydrogen are absorbed (II)²⁴⁴ On the other hand



²⁴³ A. Windau, F. v. Werdner, and H. Schneider, *Ber.* **65**, 1106 (1932)

²⁴⁴ R. Kuhn and E. F. Möller, *Angew. Chem.* **47**, 145 (1934)

titration with perbenzoic acid indicates only three of the four double linkages. But the presence of the fourth double bond can be demonstrated as follows. Upon reduction with sodium and alcohol two different dihydro compounds are formed—dihydro vitamin D_2I and II (III). Compound I has also been obtained by sodium reduction of tachysterol₂, as previously mentioned. This derivative contains three double bonds²⁴⁵ all of which react with perbenzoic acid to form a crystallized trioxide (IV)²⁴⁶. These results indicate that vitamin D_2 contains one double bond more than ergosterol which in turn means that one of the four rings present in ergosterol has been opened since vitamin D_2 contains only three rings according to its empirical formula and the number of double bonds present.

All sterols yield upon dehydrogenation with selenium the same characteristic hydrocarbon γ methyl cyclopenteno phenanthrene. In accordance with the conception that vitamin D_2 does not contain the typical four ring system of ergosterol the typical dehydrogenation hydrocarbon could not be obtained upon reaction with selenium²⁴⁷. Vitamin D_2 has this property in common with tachysterol₂ as previously described.

Of the four double bonds one is in the side chain as in ergosterol, since methyl isopropyl acetaldehyde (V) is formed upon ozonolysis of the vitamin²⁴⁸. The other three double bonds are in conjugation to each other as must be concluded from the typical absorption spectrum and its extinction coefficient. The fact that at least two of these linkages are in conjugation is shown by the formation of an addition compound of the acetate of vitamin D_2 with maleic anhydride (VI)²⁴⁹. Upon saponification the latter yields a dicarboxylic acid which by reaction with diazomethane is converted into a dimethyl ester (VII). This ester has been obtained in two isomeric forms. Upon catalytic hydrogenation of the mixture of the two esters a dihydro compound is obtained (VIII). This compound upon oxidation with ozone does not yield the methyl isopropyl acetaldehyde which proves that the double bond in the side chain has been reduced. The reaction product of the ozonolysis is however a saturated ketone of the empirical formula $C_{15}H_{24}O$ (IX) according to the analysis of its semicarbazone and its oxime. The ketone contains therefore two rings and the side chain of the vitamin D_2 , as evident from the number of hydrogen atoms present. The number of carbon atoms present reveals that the two rings contain only the original side chain and the

²⁴⁵ A. Windaus and C. Roosen Runge *Z. physiol. Chem.* 260 181 (1939).

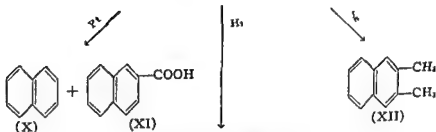
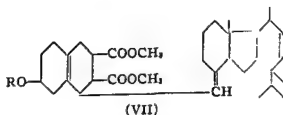
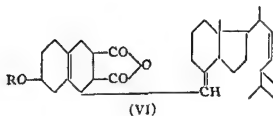
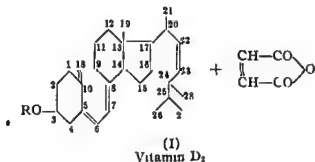
²⁴⁶ S. v. Reichel and M. D. ppe *Ibid.* 239 143 (1936).

²⁴⁷ H. Lettré *Ann.* 511 280 (1934).

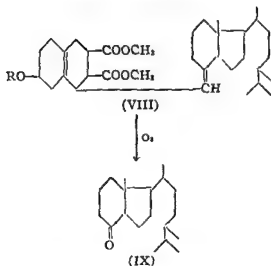
²⁴⁸ A. Cuatrecasas, Z. N. Kamaya and H. H. Imhoffen *J. Biol. Chem.* 194 116 (1932).

²⁴⁹ A. Windaus and W. Thiele *Ibid.* 521 160 (1935).

original angular methyl group between the rings C and D. The keto group is therefore situated in what was originally ring C. In ergosterol ring C is connected with ring B through carbon atoms 8 and 9. The



(Formula continued on following page)

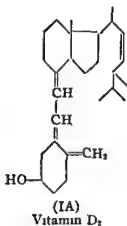


Keto group in the ozonolysis reaction product from the vitamin derivative indicates that in the vitamin molecule only one linkage exists between ring C and what was ring B in the ergosterol molecule. In addition, the formation of a ketone shows that ring C is connected with the rest of the molecule by a double bond. It follows that in vitamin D_2 ring B exists no longer but has been opened. Ring cleavage has occurred between carbon atoms 9 and 10 for a keto group at carbon atom 9 in the ozonolysis reaction product is impossible since a double bond cannot exist between carbon atoms 9 and 10 due to the fact that carbon atom 10 is quaternary in ergosterol. It is then concluded that one double bond in the vitamin D_2 molecule is in the 7,8 position. The constitution of rings C and D and of the side chain is thereby established.

The constitution of ring A and its connection with ring C through what was ring B in the ergosterol molecule has been elucidated as follows. The dicarboxylic acid (VII) obtained by maleic anhydride addition to vitamin D_2 followed by hydrolysis yields upon a platinum dehydrogenation naphthalene (X) and naphthoic acid (XI). Upon dehydrogenation of the diester of the dicarboxylic acid with selenium 2,3-dimethyl naphthalene (XII) is obtained. The methyl groups in the latter compound are derived from the carboxyl groups as can be shown by dehydrogenation of similar dicarboxylic acid esters.⁵⁰ The results of the dehydrogenation experiments must be interpreted as meaning that by addition of the maleic anhydride to the vitamin, a hydronaphthalene derivative is formed. This is possible only when the two additional unaccounted for double bonds, which

⁵⁰ W. Thiele and G. Trautmann *Ber.* 68, 224 (1935)

are in conjugation with the double bond at the 7,8 position, are in the 5,6- and 10,18 positions. The addition of the maleic anhydride occurs then on carbon atoms 18 and 6. Therefore, the constitution of vitamin D₂ is that indicated in formula (I). The formula may also be written in the form (IA)

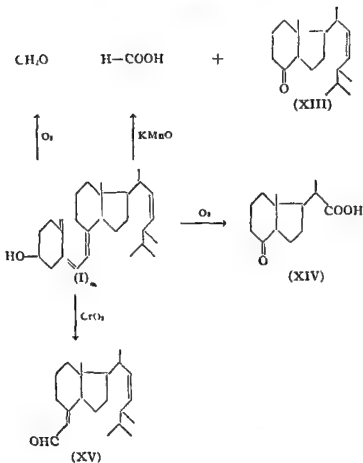


The structure of vitamin D₂ as deduced indicates the presence of a methylene group on carbon atom 10 instead of the methyl group present in ergosterol. This was furthermore proved by oxidation experiments: permanganate in acid solution yields formic acid, and ozone yields form aldehyde.²¹

These oxidation experiments give further evidence on the constitution of vitamin D₂. Permanganate oxidation yields, besides formic acid, the unsaturated ketone (XIII) which can be characterized as the semicarbazone and oxime. By selective hydrogenation of the double bond there is obtained the same saturated ketone (IX) that was found in the direct oxidation of a 22 dihydro derivative as previously described. Oxidation of the vitamin D₂ with ozone yields besides formaldehyde the keto acid (XIV), which is obtained by cleavage at the double bonds between the carbon atoms at the 7,8 and 22,23 positions. By careful oxidation of vitamin D₂ with chromic acid rupture occurs at the double bond in the 5,6 position and the doubly unsaturated aldehyde (XV) can be isolated.²² The constitution assigned to this aldehyde is in agreement with the result of its ultraviolet spectrum, which indicates the presence of an $\alpha\beta$ unsaturated oxo group.

²¹ A. Windaus and W. Grundmann, *Ann.* 521, 160 (1935).

²² I. M. Heilbron, R. N. Jones, K. M. Samant and F. S. Spring, *J. Chem. Soc.* 1936, 905.



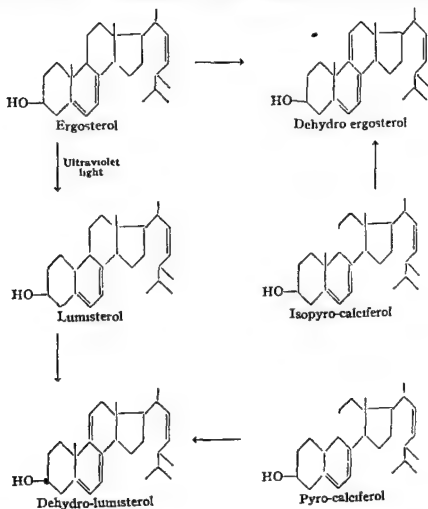
Upon heating vitamin D_2 (calciferol) in the absence of air at $160-190^\circ$ pyro calciferol and isopyro calciferol are obtained. These products are of interest in the determination of the structure of vitamin D_2 and also in the isomerisms involved in the photochemical activation of ergosterol and the destruction of the formed vitamin D . Pyro calciferol and isopyro calciferol crystallize as molecular addition products and can be separated after acetylation by fractional crystallization. Neither of the two pyro compounds shows antirachitic activity.

Analysis and molecular weight determinations indicate that the two compounds are isomers of ergosterol and of vitamin D_2 . According to the results obtained upon catalytic hydrogenation²⁵³ and according to titrations with perbenzoic acid²⁵⁴ the pyro compounds contain three double bonds. Hence a ring closure is involved in the formation of the pyro compounds.

²⁵³ M. Miller, *J. phys. Chem.* **233**, 23 (1933).

²⁵⁴ I. Busse, *ibid.* **214**, 211 (1933).

from vitamins D Upon dehydrogenation with selenium γ methyl cyclo penteno phenanthrene is obtained,²⁵³ showing that the basic sterol skeleton is present This indicates that the ring-closure occurred between the same carbon atoms that were involved in the ring cleavage during the activation process of ergosterol Of the three double bonds, one is inferred to be in the original 22,23 position The other two are in conjugation with each other since both pyro- and isopyro-calciferol react with maleic anhydride to yield addition compounds The double bonds are furthermore situated in the same ring, since upon nitric acid oxidation toluene 2,3,4,5-tetracarboxylic acid is obtained²⁵⁵ The difference between pyro and isopyro-calciferol is evident from the fact that isopyro-calciferol forms an addition compound with digitonin whereas pyro-calciferol does not Furthermore, pyro-calciferol, but not the isopyro-



isomer, forms upon dehydrogenation with eosin in the presence of visible light a bimolecular compound²⁵⁶ as does ergosterol. Isopyro-calciferol has properties very similar to those of ergosterol in contrast to the other products in the irradiation series from ergosterol. Moreover, both ergosterol and isopyro calciferol form upon dehydrogenation with mercuric acetate the identical compound, dehydro ergosterol²⁵⁷ which differs from ergosterol in an additional double bond at the 9-11 position²⁵⁸. On the other hand pyro calciferol when subjected to the same treatment yields a dehydro derivative which is identical with that obtained by dehydrogenation of lumisterol²⁵⁹. Thus it is evident that ergosterol and isopyro calciferol differ only in the spatial arrangement at carbon atom 9 while lumisterol and pyro calciferol differ only in the spatial arrangement at carbon atom 10. This series of reactions proves furthermore that the only change involved upon conversion of ergosterol into lumisterol is an epimerization of the substituents at carbon atom 10.

After the pyro calciferols were shown to be stereoisomers of ergosterol and of lumisterol it became interesting to study the changes which occur upon ultraviolet light irradiation of the pyro compounds. The results²⁶⁰ indicate that an entirely different reaction mechanism occurs. While the pyro-compounds are not stable to irradiation no indication of the existence of intermediate products is obtained by following spectroscopically the changes occurring during the irradiation. No antirachitic substance is obtained. The end products of the photochemical process are obtained in crystallized form but these compounds show no absorption spectrum in the critical region between 240 and 310 m μ . On heating the irradiation end products are reconverted into the two pyro-compounds. This suggests that a ring cleavage similar to that obtained on irradiation of ergosterol does not occur upon irradiation of pyro-calciferol and of isopyro calciferol. The changes involved appear to indicate only that a rearrangement of the two double bonds in ring B occurs so that they are no longer conjugated. Upon heating the conjugation of the double bonds is restored.

(c) The Constitution of Vitamin D₃

The constitution of vitamin D₃ which can be derived from 7-dehydro cholesterol has been inferred by analogy with the proved constitution of vitamin D₂ to be as follows (I)

²⁵⁶ T. Fennedy and F. S. Spring *J. Chem. Soc.* 1939 250

²⁵⁷ A. Windaus and K. Dimroth *Ber.* 70 376 (1937)

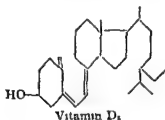
²⁵⁸ M. Möller *Z. physiol. Chem.* 231 75 (1935)

²⁵⁹ J. B. H. Ibbotson, F. S. Spring and P. A. Stewart *J. Chem. Soc.* 1935 1221

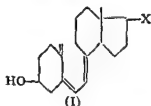
²⁶⁰ K. Dimroth *Ber.* 70 1631 (1937)

(e) *The Constitution of Vitamin D₃*

Vitamin D₃ is antirachitically activated 7 dehydro sitosterol and should therefore have the following constitution

(f) *The Constitution of Other Vitamins D*

On the basis of the known constitutions of the vitamins D₂ and D₃ the general formula of any vitamin D is believed to be represented by (I) wherein X stands for the side chain, the constitution of which varies with each member of the vitamin D group



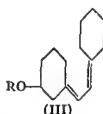
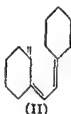
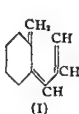
It has been previously stated that not all of the members of the naturally occurring group of vitamins D have been isolated and therefore their chemical constitution is still obscure

A new vitamin D has been isolated from cod liver oil (vitamin D₈ (7)) the chemical constitution of which is not known. Since it has a boiling point which is considerably lower than that of the rest of the vitamins D occurring in cod liver oil it has been suggested that it may not have a side chain at all or only a very small one²⁶⁵. It is of course possible that this vitamin differs from the other known ones in stereochemical arrangement at one or more of the asymmetric carbon atoms. The establishment of the structure of this new vitamin must await its isolation in crystallized form.

²⁶⁵ C. I. Bill, O. N. Massengale, K. C. D. Hickman and I. J. C. y, *J. Biol. Chem.* 126 241 (1938)

18 Synthesis

Aside from the previously discussed partial syntheses of vitamins D by activation from provitamins no vitamin D has been obtained synthetically. The total synthesis of one of the vitamins D would be of considerable theoretical interest mainly in order to study the exact stereochemical relations of the asymmetrical carbon atoms. Attempts have been made to synthesize part of the molecule, that is the system of three conjugated double bonds with the attached cyclo hexane rings. The following com-



pounds I, ⁶⁶ II²⁶⁷ and III,²⁶⁸ have been obtained by total synthesis. The antirachitic efficacy of these compounds has not been investigated.

19 Industrial Methods of Preparation

There are a number of different vitamin D preparations on the market. Some of these originate from fish liver oils which have been processed according to the methods described for the preparation of vitamin A from fish liver oils. Percomorph liver oil, cod liver oil and halibut liver oil are offered for human consumption while the oils of other fish are used for animal food.

Besides the sale of the natural product, vitamin D prepared by activation of provitamins D is also marketed. Many of the commercial products are combinations of several forms of vitamin D. The activation is carried out according to the various methods described, but especially by ultraviolet irradiation and by bombardment with low velocity electrons. Quite a number of modifications of these two processes have been patented and are actually employed. The provitamins which are used commercially have been discussed on page 365.

Commercially important are the food products which are fortified with vitamin D, such as bread (containing 460 International Units to the 24

²⁶⁶ N. A. Milas and W. I. Alderson, *J. Am. Chem. Soc.* 61, 2534 (1939).

²⁶⁷ K. Dimroth, *Ber.* 71, 1333, 1346 (1936); K. Dimroth and H. Johnson, *Ibid.* 71, 2608 (1938).

²⁶⁸ J. B. Aldersley and G. N. Burkhardt, *J. Chem. Soc.* 1938, 545.

ounce loaf) breakfast cereals margarine etc Besides the actual addition of vitamin D, some foodstuffs are themselves irradiated thus gaining anti rachitic efficacy

One of the most suitable carriers for vitamin D, as far as human nutrition is concerned is milk Milk can be fortified in its natural vitamin D content by the following methods

1 A vitamin D concentrate in oil is added to yield milk of a potency of at least 400 International Units per quart ²⁷⁰

2 A solution of vitamin D in a suitable solvent, such as propylene glycol is added

3 Cows upon irradiation for example with the sun or with artificial ultraviolet light secrete a milk of increased vitamin D potency ²⁷⁰ that has up to 40-50 International Units per quart

4 Cows are fed with vitamin D, usually with irradiated yeast The milk secreted by such cows contains metabolized vitamin D and is standardized to contain not less than 400 International Units per quart

5 The milk itself is irradiated thereby converting the provitamin D content into vitamin D This procedure which is one of the cheapest, is also one of the most difficult since an unpleasant taste and odor are easily produced by ultraviolet irradiation ²⁷¹ Quite a number of process modifications are being used and have been patented ²⁷ The irradiated milk is standardized at 135 International Units per quart but up to 200 International Units can sometimes be produced Irradiated milk is therefore of obviously low potency

For clinical use vitamin D is marketed in oil solution or in solution with a suitable organic solvent such as propylene glycol ethers of polyhydroxy alcohols for example glycerol diethyl ether and dimethoxy trioxy hexane oxalkyl ether and alkoxyethyl ether of polyhydroxy-compounds and fatty acid amides for example ethyl acetamide Colloidal solutions of vitamin D in water can be prepared that are sterilized by the application of heat

For general use by the public vitamin D is available also in the form of emulsions, tablets capsules tonics and malt preparations

20 Biogenesis

The biogenesis of the large amounts of vitamin D found in fish is a much discussed problem It seems impossible that these great quantities

²⁷⁰ T P Zucker *Am J Pub Health* 13 10 (1923)

²⁷¹ J E Camp on K M Henry S K. Kon and J Mackintosh *Biochem J* 31 81 (1937) H R Nechtel and C A Hoppert *J Nutrition* 11 537 (1936)

²⁷² A G Weckel and H C Jackson *Food Research* 3 419 (1936)

²⁷ See the review by W Diemarle *Chem Fabrik* 14 51 (1941)

originate from provitamins in the skin of fish since not enough ultraviolet radiation is available for their conversion. Furthermore, as has been proved for the catfish at least,²⁷³ fish are very sensitive to ultraviolet irradiation, and the amount of vitamin D produced under the influence of light is negligible. Since, however, ultraviolet light penetrates both fresh water and sea water for about three feet,²⁷⁴ the possibility has often been considered that vitamin D is produced in the plankton and in algae which in turn constitute the normal food supply of fish.²⁷⁵ Actually small amounts of vitamin D have occasionally been found in marine microorganisms.^{76, 77} It seems questionable, however, that this is a logical explanation of the presence of vitamin D in fish oil. The type of vitamin D in microorganisms is at least as far as is known, not the type of vitamin D found in fish, since the vitamin D from microorganisms is relatively inactive when fed to chickens, whereas the vitamin D from fish oil is generally highly active. If, then, the vitamin D present in fish actually originates from the vitamin D in microorganisms, the hypothesis must be accepted that the chemical structure of the vitamin is altered in the animal organism, which is very unlikely.

Another explanation offered is that the fish organism may contain an enzyme system which takes care of the conversion of provitamin D to vitamin D. Whereas it is certain that no such enzyme system exists in humans or in chickens, the actual presence or absence of such an enzyme system in fish has not been demonstrated.

Instead of the existence of an enzyme system, the so called mitogenetic radiation (Gurwitsch rays) has been postulated to cause the transition of provitamins D to vitamins D.²⁷⁸ It has been claimed that energy metabolism in the growing cell causes the production of ultraviolet rays of about 190-250 m μ which play a special role in the growing tissue. These rays may cause the production of vitamin D in fish and in man. Since only preliminary results have been published, it seems too early to pass any judgment on this subject. Some investigators deny the existence of the mitogenetic radiation.^{79, 280}

²⁷³ C. E. Bills, *J. Biol. Chem.* 72, 751 (1937).

²⁷⁴ H. H. Darby and H. T. Clarke, *Science* 85, 318 (1937).

²⁷⁵ H. Steenbock and A. Black, *J. Biol. Chem.* 64, 263 (1926).

²⁷⁶ H. H. Darby and H. T. Clarke, *Science* 85, 318 (1937).

²⁷⁷ A. M. Copping, *Biochem. Z.* 28, 1516 (1934); J. C. Drummond and F. R. Gunther, *J. Exptl. Biol.* 11, 203 (1934).

²⁷⁸ H. Mai, *Abhandl. aus der Kinderheilkunde u. ihren Grenzgeb.* 1937, H. 40.

²⁷⁹ K. H. Kreuchen, *Angew. Chem.* 47, 185 (1934); E. N. Harvey, *Naturwissenschaften* 12, 16 (1924).

²⁸⁰ J. Levine and A. H. Steinhaus, *Proc. Am. Physiol. Soc.* 1941, 173.

Finally the theory of a total vitamin D synthesis in fish has been brought forward. While this theory appears plausible and possible, the experimental evidences⁸¹ offered to prove this thesis are not conclusive.

The origin of vitamin D in higher animals is an entirely different problem. Higher animals are not able to synthesize vitamin D at least not in sufficient quantities as is evident by the incidence of rickets. The previously discussed mitogenetic radiation may produce extremely small amounts of this vitamin from provitamin present in the tissue. If this is the case then the susceptibility for rickets sometimes observed in infants may be explained^{82, 283} on the basis of a failure to produce enough radiation or on the basis of an absence of provitamins.

There is no question but that normally a considerable amount of vitamin D is gained by exposure of the animal and human body to the sun. It is assumed that the vitamin D thus formed is absorbed into the blood stream. However the mechanism of this reaction is not clear. The skin of various organisms—of humans^{284, 285} of cattle²⁸⁶ of pigs²⁸⁷ etc.—has been shown experimentally to contain provitamin D in an amount which is at least 10 and probably 100 times greater than the amount found in the inner parts of the body. Attempts have repeatedly been made to determine how deep ultraviolet radiation from the sun is able to penetrate through the epidermis but no conclusive data have been obtained. While most workers believe that ultraviolet rays penetrate human skin only slightly that is 0.1 mm²⁸⁸ it has been argued that the depth of penetration depends on viability and may go through living tissue for 1.2 mm.⁸⁹ It has also been stated that provitamin D may be secreted by the sebaceous glands and that the vitamin D after its formation is absorbed by the skin. That the human skin is able to absorb vitamin D has repeatedly been shown²⁹⁰ but it is unlikely that the sebaceous glands excrete provitamin D. It has however been reported⁹¹ that thorough washing of human skin and subsequent irradiation failed to provide enough activation to prevent serious deficiency. This observation requires confirmation however.

⁸¹ C. T. Bliss, *J. Biol. Chem.* 72, 71 (1927).

H. Mai, *Abhandl. u. d. der Akad. der Wissenschaften u. ihren Grenzgeb.* 1937, H. 4.

⁸² I. Glinmann, *Ergeb. Vitam. u. Horm. Forsch.* 1, 8 (1938).

²⁸⁴ A. F. Heas and M. Weinlock, *J. Biol. Chem.* 64, 181 (1925).

²⁸⁵ H. Hentchel and I. Schindl, *Abh. Hochsch.* 9, 262 (1930).

²⁸⁶ A. F. Heas and M. Weinlock, *J. Biol. Chem.* 64, 181 (1925).

²⁸⁷ A. Windaus and F. Bock, *Z. physiol. Chem.* 245, 168 (1936).

²⁸⁸ J. Clark, *Physiol. Rev.* 2, 7 (1927).

²⁸⁹ W. T. Anderson and D. I. Macht, *Am. J. Physiol.* 85, 30 (1928).

²⁹⁰ M. F. Foder, *Z. Vitaminforsch.* 3, 241 (1934). A. C. Helmer and C. Jansen, *Studies Inst. Derm.* 7, 1, 81, 91 (1937).

⁹¹ A. C. Helmer and C. Jansen, *Studies Inst. Derm.* 7, 1, 207 (1937).

In birds the production of vitamin D by sunlight is apparently still more complicated. It has been suggested^{297, 298} that the preen gland excretes a provitamin D containing oil which is distributed over the feathers and after exposure to sunlight is either ingested or absorbed through the skin. This could, however, not be proved experimentally²⁹⁴. Furthermore, no provitamin D could be detected spectroscopically in the sterol fraction from preen glands²⁹⁵. It has furthermore been shown that irradiation of the feet of chickens without preen glands cured rickets,²⁹⁶ and the presence of provitamin D in the feet has been proved²⁹⁵.

The site of vitamin D formation in fur bearing animals is also largely unknown. It has been claimed²⁹⁷ that rats which are prevented from licking their fur develop rickets. On the other hand, the skin of rabbits was shown to be antirachitic, the dorsal skin more so than the ventral²⁹⁸ but only in normal animals, not in those suffering from rickets.

The formation of vitamin D in the organism is subject to seasonal variation due to the seasonal change of sunlight. Thus the incidence of rickets in the northern hemisphere is the greatest from January to March. Similar seasonal changes can also be demonstrated in the vitamin D content of milk²⁹⁹ and of eggs³⁰⁰ which contain maximum amounts during the summer months and minimum amounts during the late winter months.

21 Specificity

In discussing the specificity of vitamins D a differentiation is made between 'compound specificity' and 'species specificity'. Compound specificity is the response to various forms of vitamin D by one given species. Species specificity is the efficacy of one given form of vitamin D for various species.

The basis for all statements regarding specificity or efficacy of vitamin D is the rat test. Each form of vitamin D has a certain number of rat units per gram of the pure product. These rat units are expressed in International Units or in U. S. Pharmacopoeia Units which are based on the efficacy of vitamin D₂. This method of referring to the International

²⁹⁷ H. C. Hou *Chinese J. Physiol.* 2: 345 (1928); 3: 171 (1929); 4: 79 (1930); 5: 11 (1931).

²⁹⁸ W. Rowan *Nature* 121: 323 (1928).

²⁹⁹ H. R. Knowles, E. B. Hart and J. G. Halpin *Poultry Sci.* 14: 33 (1935).

³⁰⁰ H. R. Rosenberg unpublished experiments.

²⁹⁴ H. C. Hou *Chinese J. Physiol.* 2: 345 (1928); 3: 171 (1929); 4: 79 (1930); 5: 11 (1931).

²⁹⁵ E. Reckling *Strahlentherapie* 25: 568 (1927).

²⁹⁶ H. C. Hou and E. Tso *Chinese J. Physiol.* 4: 93 (1930).

²⁹⁷ J. E. Campion, K. M. Henry, S. K. Kon and J. Mackintosh *Biochem. J.* 31: 81 (1937). H. L. Bechtel and C. A. Hoppert *J. Nutrition* 11: 537 (1936).

²⁹⁸ G. M. DeVaney, H. E. Munsell and H. W. Titus *Poultry Sci.* 12: 215 (1933).

Unit of vitamin D is however only justified in a discussion regarding the compound specificity. For the determination of the 'species specificity' the International Units proved to be inadequate since vitamin D₂ has a very low efficacy on chicks. There appears to exist a very definite species specificity at least for the organisms which have been investigated. Thus, one rat unit is not necessarily one chick unit or turkey unit or man unit. The difference between rat units and man units appears to be only slight. The difference between rat units and chick units is, however, very significant. In the following table the compound and species specificity are summarized for the vitamins which have been investigated.

TABLE VI
COMPOUND AND SPECIES SPECIFICITY OF KNOWN VITAMINS D

Provitamin D	Vitamin D	Rat efficacy of pure vitamin D per gram in Standard Units (1 S U = 1 million I U)	Chick efficacy as % of rat activity
Ergosterol	Vitamin D ₂	40 S U	1-3
Epi ergosterol		Unknown but active	?
7 Dehydro cholesterol	Vitamin D ₃	40 S U	100
Lpi 7 dehydro cholesterol		4 S U	?
22 Dihydro ergosterol	Vitamin D ₄	20-30 S U	20 ¹⁰¹
7 Dehydro sitosterol	Vitamin D ₄	13 S U	Less than 100 ¹⁰²
?	Vitamin D ₄ (?)	?	20-50
7 Dehydro stigmasterol		0.1 S U	?
22,23 Oxido ergosterol		Only feebly active ¹⁰³	?
Mussel provitamin D		40 S. U ¹⁰⁴	100 ¹⁰¹ ¹⁰⁵
Δ^{17} Androsta-diene diol			
317		0.1 S U ¹⁰⁶	
3 Hydroxy Δ^{17} cholanolic acid		0.1 S U ¹⁰⁷	
	Dihydro-tachysterol	0.2 S U ¹⁰⁸	

¹⁰¹ P. G. McDonald *J. Biol. Chem.* 114: 155 (1936)

¹⁰² W. Grab *J. physiol. Chem.* 243: 63 (1936)

¹⁰³ A. W. Naudus, Linsal and K. Buchholz quoted by K. D. Mroth and J. Paland *Ber.* 72: 187 (1939)

¹⁰⁴ A. G. Boer, J. van Niekerk, E. H. Reerink and A. van Wijk *U.S.P.* 163: 659

¹⁰⁵ J. van Niekerk and F. Franken *Acta B. erica Neerland. Physiol. Pharmacol. Microbiol.* 8, 13 (1938)

¹⁰⁶ A. Butenandt, F. Hausman, J. Paland, D. von Dresler and U. Meertens *Ber.* 71: 1316 (1938)

¹⁰⁷ K. D. Mroth and J. Paland *ibid.* 72: 187 (1939)

¹⁰⁸ G. A. D. Haslewood *Biochem. J.* 33: 404 (1939) *J. Chem. Soc.* 1938: 24

¹⁰⁹ F. v. W. rder *J. physiol. Chem.* 260: 119 (1939)

Compound Specificity

The results of the compound specificity tests need further interpretation. It will be recalled that none of the activation intermediates is antirachitic effective at least in the amounts investigated. Thus, in order to obtain vitamin D activity ring B of the molecule must be opened between C₉ and C₁₀ and the double bonds must be in the correct positions. The latter is evident from the inactivity of tachysterol.² The most revealing result is the relatively great specificity of rats toward small differences in the vitamin D molecule. Whereas vitamins D₂ and D₃ are equally active in rats, hydrogenation of the side chain double bond of vitamin D₂, which results in the formation of vitamin D₄, decreases the activity considerably. On the other hand, activated 7 dehydro stigmasterol which differs from activated ergosterol by one additional methyl group in the side chain, is practically devoid of activity. Activated 7 dehydro sitosterol, which differs from activated 7 dehydro stigmasterol only in having a saturated side chain and which differs, therefore, from activated 22 dihydro ergosterol in one additional methyl group in the side chain is somewhat more active. 22,23 Oxido ergosterol shows only feeble activity, while the total absence of the side chain as in activated $\Delta^{5,7}$ androsta diene-diol 3,17³⁰⁹ or the presence of a four membered carboxyl group containing side chain as in the bile acid analog of vitamins D (activated 3 hydroxy $\Delta^{5,7}$ chola dienic acid)³¹⁰ makes the compounds practically inactive. It is thus evident that considerable compound specificity rests in the structure of the side chain. An epimerization of the hydroxyl group in vitamin D causes a considerable decrease in efficacy, although the activity is not entirely lost ($1/10$ in the case of activated epi 7 dehydro-cholesterol). Vitamins D are furthermore only active when the hydroxyl group is free. Esters and ethers of vitamins D are inactive.³¹¹ Those esters which can be hydrolyzed in the organism are active.

It has repeatedly been observed that vitamin D given in milk exerts a greater antirachitic response than when the same amount of vitamin D is given in oil solution. While it is now believed³¹² that this effect is brought about by an optimum simultaneous intake of phosphorus and calcium as present in milk it was stated that a synergistic factor may

³⁰⁹ A. Butenandt, E. Hausmann, J. Paland, D. von Dresler and U. Meinerts, *Ber.* **71**, 1316 (1938); K. D. Mroth and J. Paland, *Ibid.* **72**, 187 (1939).

³¹⁰ G. A. D. Haslewood, *Biochem. J.* **33**, 454 (1939); *J. Chem. Soc.* 1938, 24.

³¹¹ A. Windaus and O. Rygh, *Nachr. Ges. Wiss. Göttingen, Math. physik. Klasse* **III**, 202 (1938).

³¹² B. O'Brien and K. Morgareidge, *J. Nutrition* **16**, 91, 395 (1938); *J. Biol. Chem.* **128**, LXXXV (1939).

be involved. A symplex formed from vitamin D and lactalbumin was held responsible for the alleged enhanced antirachitic activity³¹³

A few compounds which evidently have not the basic structure of the vitamins D have also been found to exhibit antirachitic activity. Thus in rats the addition of iodine or iodides to the rachitogenic diet prevents or cures rickets³¹⁴. Certain organic acids especially tartaric and citric acid³¹⁵ are able to prevent or cure rickets in rats but not in chicks³¹⁶. These effects are closely related to the acid base properties of the diet and its influence upon the development of rickets (see page 425). It has been repeatedly shown that upon treatment of cholesterol with various chemicals a compound or compounds are obtained which (without further activation) exhibit slight but definite antirachitic activity when tested on rats and which are reported to show a chicken effectiveness of over 100%. The chemistry of these products is entirely unknown. The activation is confined to sterols is somewhat specific to the constitution of the sterol employed³¹⁷ and does not materially activate the provitamins D. The reaction is brought about by fuller's earth³¹⁸ and by sulfuric acid³¹⁹. The active compound was believed to be a monosulfonation product of cholesterol or cholesterilene. However cholesterilene sulfonic acid was subsequently found to be inactive³²⁰. Active products were obtained by the treatment of sterols with KHSO_4 , CuSO_4 , ZnCl_2 , AlCl_3 , P_2O_5 , trichloro acetic acid, phosphoric acid etc.³²¹

Species Specificity

The *chick* is considerably more sensitive than the rat as is evident from Table VI. Only a few vitamins D have been tested on chicks. Vitamin D_3 and the vitamin derived from mussel provitamin³²² are highly active while vitamin D_2 is only slightly active. The vitamins D_4 and D_6 (?) also have a definite but slight chicken activity. As has been pointed out before

³¹³ C. C. Supplee, S. Ansbacher, R. C. Bender and C. T. Flanagan, *J. Biol. Chem.* 114, 95 (1936).

³¹⁴ R. L. Coq, *Compt. rend.* 204, 1801 (1937); R. L. Coq and R. Duffan, *Compt. rend. soc. biol.* 128, 619 (1938).

³¹⁵ B. Hamilton and C. Schwartz, *Am. J. Diseases Child.* 46, 669 (1933); B. Hamilton and M. M. Dewar, *Ibid.* 54, 548 (1937); A. T. Shohl, *J. Nutrit.* 14, 69 (1937).

³¹⁶ J. T. Correll, *J. Nutrit.* 21, 515 (1941).

³¹⁷ J. C. Eck and B. H. Thomas, *J. Biol. Chem.* 128, 757 (1939).

³¹⁸ C. E. B. Ellis and F. G. McDonald, *Ibid.* 68, 821 (19 6); S. K. Kon, F. Daniels and H. Stenbock, *J. Am. Chem. Soc.* 50, 2573 (1928).

³¹⁹ L. Yoder, *Science* 80, 385 (1934); L. Yoder, B. H. Thomas and M. Lyons, *J. Nutrition* 9, Suppl. 6 (1935); L. Yoder, *J. Biol. Chem.* 115, 71 (1936); J. C. Eck, B. H. Thomas and L. Yoder, *Ibid.* 117, 635 (1937); J. C. Eck and B. H. Thomas, *Ibid.* 119, 621 (1937).

³²⁰ A. Windaus and P. Kuhr, *Ann.* 532, 57 (1937).

³²¹ J. C. Eck and B. H. Thomas, *J. Biol. Chem.* 119, 631 (1937); 128, 67 (1939).

³²² J. van Nierkerk and T. Franken, *Arch. Biochem. Biophys.* 13, 13 (1938).

in the general discussion on vitamins D (page 384), the "chicken activity" is a relative figure indicating the efficiency of a given vitamin D when fed on the basis of rat units and compared with the same number of rat units of a "U S Reference Cod Liver Oil," the chicken activity of which has arbitrarily been chosen to indicate 100% activity. In this connection it is also interesting to compare the chicken activity of the vitamin D in various fish liver oils with the cod liver oil (C L O) as is done in Table VII. The probable experimental error (PE) involved in the determinations is also given.

From Table VII it is evident that the natural fish vitamin D is a mixture of vitamins D and the conclusion has been drawn that at least one more vitamin D exists than is known today, since chicken activities as high as 200% and 300% have been observed.

The *turkey* appears to have a species specificity which differs from that of chicks. Although the difference between the chicken and turkey activity is considerably less than the difference between chicken and rat activity, it must be concluded that a chick unit of vitamin D is not necessarily identical with a turkey unit of vitamin D.^{323 324} The term 'turkey unit' is used in analogy with the term 'chick unit' and means the biological response of one International Unit of vitamin D from U S Reference Cod Liver Oil.

The efficiency with which *man* utilizes various forms of vitamin D is much more difficult to establish. There does not seem to be any question but that man can utilize vitamin D from fish liver oil, vitamin D₂ and vitamin D₃ when administered *per os*. None of the other forms of vitamin D has been investigated. On the basis of the available experimental data it is believed that when given by mouth vitamin D₃ is more active perhaps 1.5 times, than vitamin D₂.³²⁵ This is especially well demonstrated with babies in the shock therapy,^{326 327 328} and has also been repeatedly observed by feeding infants daily amounts of vitamin D prophylactically^{329 330 331} and curatively,³³ although some investigators believe

³²³ T. H. Jukes and T. D. Sanford *J. Nutrition* 18: 71 (1939).

³²⁴ H. M. Scott, J. S. Hughes and H. W. Loy *Poultry Sci.* 11: 177 (1932). F. D. Baird and D. J. Greene *Ibid.* 14: 70 (1935).

³²⁵ P. C. Jeans *J. Am. Med. Assoc.* 106: 2066-2150 (1936).

³²⁶ E. Graser *Z. Kinderheilk.* 61: 716 (1940).

³²⁷ H. J. Hartenstein *Monatsschr. Kinderheilk.* 76: Nos. 3 and 4 (1937).

³²⁸ G. O. Harnapp *Ältn. Wochschr.* 17: 390 (1938).

³²⁹ A. F. Hess, J. M. Lewis and H. Rivkin *J. Am. Med. Assoc.* 94: 1885 (1930). A. F. Hess and J. M. Lewis *Ibid.* 99: 647 (1932); 101: 181 (1933).

³³⁰ H. Brockmann *Klin. Wochschr.* 16: 1383 (1937).

³³¹ T. G. H. Drake, F. F. Tisdall and A. Brown *J. Pediatrics* 9: 421 (1936).

³³ J. M. Lewis *J. Pediatrics* 10: 155 (1937). J. S. Hood and I. Ravitch *Ibid.* 11: 521 (1937).

TABLE VII
RELATIVE EFFECTIVENESS OF VITAMIN D FROM DIFFERENT SOURCES FOR
RATS AND CHICKENS

Oil No	Name of fish (or sterol)	Vitamin as given per 100 gm diet			Response obtained			Efficacy ratio	
		A	D		Femur ash %	I U of D/100 gm	PE %	C L O = 100	PE %
		I U	I U	PE %					
1	Hahbut	510	17.5	7	45.4	15.1	+9 -9	86	+11 -11
2	Round nosed sole	1900	18.9	6	45.57	15.4	9 9	81	11 11
3	Tuna bluefin (Cal)	13	7.2	7	35.98	*			*
3	Tuna bluefin (Cal)	110	57.6	7	42	3.3	8 8	16	11 11
3 x	Tuna bluefin (Cal)	41	4.4	7	41.03	7.4	9 8	17	11 11
3 x	Tuna bluefin (Cal)	81	84.9	7	45.92	16	8 8	19	11 11
7 a	Tuna New England	39	7.9	7	39.99	6.4	10 10	81	12 12
7 a	Tuna New England	46	9.2	7	41.45	8.0	9 9	87	11 11
12	Albacore	9	20.0	5	44.42	12.6	9 9	61	10 10
12	Albacore	27	61.4	5	46.80	19.7	10 9	32	11 10
13	Tuna yellowfin	40	10.7	7	45.23	14.6	9 9	136	11 11
14	Tuna striped	7	9.6	0	35.62	*		*	*
14	Tuna striped	43	56.5	5	45.12	14.3	9 9	21	10 10
15	California bonito	20	8.4	6	34.31				
15	California bonito	140	58.0	6	45.93	16.5	10 9	28	12 11
16	California mackerel	630	9.8	7	44.01	11.8	10 9	120	12 11
17	Swordfish	180	10.1	6	45.53	15.3	9 9	151	11 11
18	Black sea bass	930	7.8	7	41.98	8.5	9 8	109	11 11
19	Cabrilla	8600	11.0	7	40.83	7.2	9 9	65	11 11
20	White sea bass	2	2.8	7	47.19	8.8	9 8	314	11 11
20	White sea bass	55	6.0	7	47.74	15.9	9 9	265	11 11
21	Totunava	470	10.0	7	34.90	*		*	*
21	Totunava	2800	50.8	7	44.47	12.7	9 9	21	11 11
22	Sablefish	1600	6.8	7	43.59	11.0	10 9	162	12 11
22	Sablefish	2300	9.8	7	43.71	15.0	9 9	159	11 11
23 a	Langcod	3000	1.8	6	4.66	15.7	9 9	99	11 11
24	Bocaccio	330	8.7	6	44.46	12.7	9 9	146	11 11
25	Chil pepper	4200	7.2	7	30.01	8.4	10 10	89	12 12
26	Wolfish	680	9.9	7	43.08	10.1	10 9	102	12 11
27	Basking shark	Nil	2	7	41.94	8.4	9 8	162	11 11
28	Dogfish	5800	4.0	7	47.50	9.2	9 9	230	11 11
29	Pollack	210	8.0	7	38.12	4.3	13 13	51	15 13
30	Hake	160	8.7	6	43.91	11.6	10 9	133	12 11
31	Sardine	270	10.7	7	43.96	11.7	10 9	109	12 11
Control	Cod	310	10.0	2	42.97	9.9	10 9	94	10 9
Control	Cod	2.0	8.0	2	42.08	8.6	9 8	108	9 8
Control	Cod	0	8.0	0	40.89	7.3	9 9	91	9 9
Control	Cod	250	8.0	2	41.3	8.0	9 9	100	9 9
Control	Cod	130	4.0	2	38.81	5.1	11 11	128	11 11
Control	Mai cod	0	0.0	0	35.12	0.0		*	*
Sterol	Irr ergosterol	0	00.0		40.00	6.4		3.0	
Sterol	Irr ergosterol	0	403.0		43.30	10.4		2.6	
Sterol	Irr ergosterol	0	1800.0		46.50	18.0		1.0	
Sterol	Irr cholesterol (ordinary from spinal cord)	0	8.0	7	41.72	8.2	9 8	103	11 11
Sterol	Irr 7-dehydro-cholesterol	0	13.0	6	43.93	11.7	10 9	90	12 11

Response too low for significant interpretation.

From C. E. Bills, O. N. Massengale, M. Imboden and H. Hall, *J. Nutrition* 12: 442 (1937)

that vitamin D₂ may be equally effective^{333 334} There are also observations to the effect that in the treatment of infantile rickets vitamin D₂ is not effective when administered intramuscularly, whereas vitamin D₃ is effective when applied in this manner³³⁵

22 Determination

(a) Physical Methods

There is only one physical method which has been recommended for the determination of vitamins D, namely, the *measurement of the characteristic absorption spectrum* in the ultraviolet³³⁶ The vitamins D have a maximum at 265 mμ This method is of course accurate only in the absence of compounds which have a similar absorption spectrum and, therefore cannot be applied for the determination of vitamins D in fish oils or in crude irradiation products of provitamins In the case of almost pure, crystalline vitamins D the spectroscopical method can be used To determine vitamins D in fish oils it has been recommended³³⁷ to first saponify the material and to isolate the unsaponifiable part, then to separate the vitamins D from inactive sterols and from the vitamin A present by selective adsorption on aluminum oxide The vitamins D present in the residual material is then determined spectroscopically While this method may give approximate values, the accuracy is not too great due to the losses which are unavoidable during the processing

(b) Chemical Methods

There is no chemical method by which vitamins D can be determined accurately or at least as accurately as by biological methods A number of color reactions for vitamins D have been proposed and have been used advantageously from time to time

1 **Aluminum-Chloride Color Reaction**³³⁸ Vitamins D in mixture with pyrogallol in benzene solution develop a deep violet color upon heating with aluminum chloride in alcohol This method is applicable to solutions of pure vitamin D Tachysterol and suprasterol I also give this reaction

³³³ M M Eliot & M Nelson *D J Barnes* 1 A Browne and R M Jens *J Pediatrics* 9 17 (1936)

³³⁴ N Morris and M M Stevenson *Lancet* 237 876 (1934)

³³⁵ A Nitschke *Z Kinderheilk* 61 385 (1940)

³³⁶ F H Reerink and A van Wijk *Chem Weekblad* 1932 64 H Töpelmann and W Schuhknecht *Z Vitaminforsch* 4 11 (1935)

³³⁷ I Marcus *en Dansk Tidsskr* 13 141 (1939)

³³⁸ W Halden *Naturwissenschaften* 24 296 (1936) W Halden and H Tzon *Nature* 137 301 (1936) H Tzon *Biochem Z* 287 18 (1936)

but the color developed is somewhat weaker. Vitamins D in oil solution cannot be determined by this method.

2 Antimony-Trichloride Color Reaction ³³⁹ Vitamins D give with a saturated solution of antimony trichloride in chloroform a yellow color with a characteristic absorption maximum at 500 m μ . The color developed is determined spectroscopically. This method can be used for vitamin D preparations in oil and is also fairly accurate in the presence of vitamin A. Tachysterol gives the same color and some other sterols give similar reactions but the color developed is weaker, and the absorption maximum is somewhat different. This method has been used successfully for the determination of vitamin D preparations of natural origin and is applicable for amounts as little as 0.02–0.4 mg. ^{340 341 342 343} A modification of this method in which acetyl chloride is used in addition to the other reagents employed is said to be more accurate. ³⁴⁴ It has also been recommended to free the solutions from sterols present by precipitation with digitonin and to absorb selectively the vitamin A present on Montana earth. ³⁴⁵

3 Aniline-Color Test ³⁴⁶ Liver oils and irradiated provitamins D give a red color with a mixture of aniline and hydrochloric acid.

4 Fuchsin-Sulfurous Acid Color Reaction ³⁴⁷ Crystalline vitamin D acquires a violet color when added to fuchsin sulfurous acid.

5 Phosphorus Pentachloride Color Reaction ³⁴⁸ Vitamin D in oil solution develops a reddish brown color with PCl₅. The color gradually becomes darker and is finally almost black. This reaction is non specific and has even been claimed to indicate all vitamins and hormones. ³⁴⁹

6 Tortelli-Jaffé Reaction ^{350 351 352} See description under Determination of Provitamin D (page 367).

³³⁹ H. Brockmann and Y. H. Chen / *physiol. Chem.* 241 129 (1936).

³⁴⁰ H. Brockmann and Y. H. Chen *ibid.* 241 123 (1936).

³⁴¹ J. K. Wolff / *Vitaminforsch.* 7 277 (1938).

³⁴² A. Fimmerle and M. van Pékelen / *Acta Biophysica Neerlandica Physiol. Pharmacol. Med. Biol.* 6 113 (1938).

³⁴³ K. Rittert / *Ernährungs-Jahrbuch* 32 27 (1938).

³⁴⁴ C. H. Nield, W. C. Russell and A. Zimmerli / *J. Biol. Chem.* 136 73 (1940).

³⁴⁵ J. K. Wolff / *Vitaminforsch.* 7 277 (1938).

³⁴⁶ M. J. Shear / *Proc. Soc. Exptl. Biol. Med.* 23 546 (1925).

³⁴⁷ A. Stegmann / *Kolloid Z.* 45 165 (1928).

³⁴⁸ W. Stoeltzner / *Mitt. ch. Med. Wochenschr.* 75 1584 (1928).

³⁴⁹ F. Christensen / *ibid.* 75 1883 (1928).

³⁵⁰ M. Tortelli and E. Jaffé / *Ann. chim. appl. cat.* 2 80 (1914).

³⁵¹ F. P. Häussler and E. Brauchli / *Helv. Chim. Acta* 12 187 (1929). J. M. H. Hron and J. Spring / *Biochem. J.* 24 133 (1930). V. A. Petrow, O. Rosenheim and W. W. Stirling / *J. Chem. Soc.* 1938 677.

³⁵² U. Westphal / *Ber.* 72 1243 (1939).

(c) *Biological Methods*

The biological methods for the determination of vitamins D are reliable when properly conducted, and are much superior to all physical and chemical assay methods developed so far. Furthermore the amount of vitamin D needed for biological determinations is relatively small, whereas the amount needed for chemical determinations is of an entirely different order of magnitude.

Rats and chicks are used as test animals for vitamin D determinations. All preparations to be standardized are usually first assayed on rats. The chick test takes longer time and is more costly. On the other hand, as pointed out previously, the rat efficacy is no measure of the chicken efficacy of any vitamin D preparation of unknown chemical composition. It is, however, possible to determine once and for all the rat-chicken efficacy ratio of vitamins D prepared by activation of known provitamins. The ratio of such vitamins D is constant when tested under standardized conditions.

The rat as such is not an ideal animal for vitamin D assays. Its normal needs for this vitamin are extremely small. In order to induce rickets in rats they must be fed a ration which contains calcium and phosphorus in an abnormal proportion. Usually a high calcium and low phosphorus diet is used, for example, a ratio of about 5:1. It is impossible to produce rickets with a calcium-phosphorus ratio of 1:1 in the diet, but a high phosphorus and low calcium diet may also be employed. The absolute amounts of these minerals in the diet are of equal importance³⁵³ (see page 421).

The chick needs considerable amounts of vitamin D even if the calcium-phosphorus ratio may be normal—about 1.5:1. In conducting chick experiments it is important to avoid exposure of the birds to ultraviolet light since even small amounts of these rays cause the disappearance of the deficiency symptoms.

1. **The Rat Assay Method** This method is the basis of all determinations of vitamin D and is accepted by all the leading national^{353a} and international organizations concerned with vitamin D assays. Young, growing rats are placed on a suitable rachitogenic diet (for example, Steenbock and Black diet 2965³⁵⁴ or McCollum diet 3143³⁵⁵) and groups of rats

³⁵³ H. B. Brown, A. T. Shohl, E. E. Chapman, C. S. Rose and E. M. Sauerwein, *J. Biol. Chem.* 98: 207 (1932). A. Querido, *Arch. neerland. physiol.* 20: 487 (1933). A. T. Shohl and S. B. Wolbach, *J. Nutr.* 11: 275 (1936).

^{353a} The United States Pharmacopoeia XI-1939 Supplement specifies a rat test for vitamin D assays. The assay period of this procedure is eight days; the animals receiving the vitamin D only during the first six days. The effect is evaluated according to the line test.

³⁵⁴ H. Steenbock and A. Black, *J. Biol. Chem.* 64: 263 (1925).

³⁵⁵ E. V. McCollum, N. Simmonds, P. G. Shipley and E. A. Parks, *Ibid.* 51: 41 (1922); 47: 507 (1921). *Proc. Soc. Exptl. Biol. Med.* 18: 275 (1921). *Am. J. Hyg.* 1: 492 (1921).

(7 to 10) are fed various amounts of the vitamin D to be tested while one group is used as reference group and is fed with U S Standard Cod Liver Oil After a period of from 6 to 10 days the animals are killed and the degree of rickets is determined by any one of the following methods

(a) *The "Line Test"* This is a curative method and for this purpose either the proximal end of the tibia³⁵⁶ or the distal end of the radius or ulnae³⁵⁷ is used The bones are removed from the animal and cleaned from adherent tissue A longitudinal median section is made and the section is immersed in a 2% aqueous solution of silver nitrate for one minute whereby the calcium phosphate present is converted into silver phosphate After cleaning with distilled water, the sectioned surface of the bone is exposed in water to actinic light until the calcified areas have developed a clearly defined black stain The criterion of healing is the development of a line of new calcification through the rachitic metaphysis This is determined either by visual or by photographic³⁵⁸ examination of the stained section

(b) *Ash Content of the Bone*³⁵⁹ This method utilizes the estimation of the ash content of the bones of the hind limbs of rats maintained on prophylactic levels of vitamin D

(c) *Radiographic Method*³⁶⁰ In this test the bones of rats are examined radiographically This test is used for curative and prophylactic assays

(d) *Test for Increase of Body Weight*³⁶¹ This method is based on the increase of body weight observed on young rats on a rachitogenic diet supplemented with vitamin D (originally irradiated ergosterol) This method has not been used widely, since it is not sufficiently accurate

2 **The Chick Assay Method** The best method developed so far for the determination of vitamin D in chicks is the one recommended by the Association of Official Agricultural Chemists and is based upon the use of the percentage ash content of the tibia determined under standard conditions^{362, 363} A special radiographic method has also been sug

³⁵⁶ C. E. Billa *J Biol Chem* 90 619 (1931)

³⁵⁷ F. J. Dyer *Quart J Pharm Pharmacol* 4 503 (1931)

³⁵⁸ P. F. Bech *Dansk Tidsskr Farm* 13 253 (1939)

³⁵⁹ H. Steenbock and A. Black *J Biol Chem* 61 405 (1924)

³⁶⁰ R. B. Bourdillon, H. M. Bruce, C. Fischmann and T. A. Webster *Med Research Council B 1 Special Rept Series* 1931 No 158

³⁶¹ K. H. Coward, K. M. Key and G. E. Morgan *Biochem J* 26 1585 (1937)

³⁶² *J Assoc Official Agr Chem* 20 72 (1937) W. B. Grien *J Assoc Official Agr Chem* 21 607 (1938) *Methods of Analysis A.O.A.C.* 371 (1940)

³⁶³ O. N. Masingale and C. E. Billa *J Nutrition* 12 429 (1936) W. Grab *Z physiol Chem* 243 63 (1936)

gested^{364 365} which makes use of the differences in the tarso metatarsal distances in the joints of the legs

The relative chicken efficacy of vitamin D preparations is determined³ by assaying the material on chicks on the basis of its rat unit content. The efficacy ratio for rats and chicks varies with the degree of calcification produced and is not a constant.

23 Standards

The International Unit of Vitamin D was defined in 1934 by an International Vitamin Conference held by the League of Nations.³¹⁷ One International Unit is defined as 0.025 γ of pure crystalline vitamin D dissolved in one milligram of olive oil. The properties of crystalline vitamin D₂ are defined

Calciferol or vitamin D₂ C₂₈H₄₄OH

(a) Colorless acicular crystals odorless M p 114.5–117° C (open capillary)

(b) Specific rotation

in alcohol $[\alpha]_D^{25} \approx +101^\circ$ to $+102.5^\circ$

$[\alpha]_{5461}^{20} \approx +119$ to $+122$

in chloroform $[\alpha]_D^{20} = +52^\circ$

$[\alpha]_{5461}^{20} = +62^\circ$

(c) Absorption spectrum in alcohol or other suitable non absorbing solvent : smooth curve with a maximum at 265 m μ $E_{1\text{cm}}^{1\%} = 470\text{--}485$

This standard was recommended for adoption when the International Standard solution prepared according to the recommendation of the International Vitamin Conference in 1931 becomes either exhausted or unsatisfactory. The 1931 Standard, although still available, should no longer be used since it gives different results on evaluation on rats by the line test method and by the bone ash method.³¹⁸

The International Unit is also the basis for the U. S. Pharmacopoeia Unit and in England for the M. R. C. Unit (Medical Research Council Unit). For general use in the United States a cod liver oil preparation has been set up to serve under the term *U. S. P. Reference Cod Liver Oil* for comparative studies. The vitamin D content of this U. S. Pharmacopoeia Reference Cod Liver Oil has been tested carefully against

³¹⁷ N. Olsson *Arch. Geflügelkunde* 10: 11 (1936); *Kl. Fyros afiska Sällskapet's Lund Forh. di ngr* 9: 1 (1936).

³⁶⁴ A. Z. Baker and M. D. Wright *Analyst* 65: 376 (1940).

³⁶⁵ O. N. Massengale and C. E. Bills *J. Nutrition* 12: 429 (1936).

³¹⁸ League of Nations Health Organization, Memorandum on the International Standard for Vitamin D and Its Application, March 1935, No. 30.

³⁶⁶ N. I. Cridgeman, H. Lees and H. Wilkinson *Analyst* 65: 493 (1940).

the International Standard. The latter measures properly only the activity of vitamin D₂. It has been pointed out before that birds are not able to utilize vitamin D₂ but require fish liver oil or vitamin D₃. The Association of Official Agricultural Chemists has therefore introduced the 'A O A C Chick Unit,' which according to its definition³⁶⁹ is equal in biological activity for the chick to one unit of vitamin D in the U S Pharmacopoeia Reference Cod Liver Oil when determined according to specified conditions (see page 415).

A standard such as the U S Pharmacopoeia Reference Cod Liver Oil while it serves its purpose satisfactorily can of course only be recognized temporarily. There are a number of indications that the chicken activity of cod liver oil is not the same for various batches of the oil. Ultimately this oil which contains an unknown mixture of vitamins D should be replaced by a pure crystalline vitamin D, preferably vitamin D₃. One rat unit of this vitamin is equal to one chick unit when determined according to the method specified by the A O A C.

A number of other units of vitamin D have been used or are still in use. They may be summarized in the following table.

1 International Unit	= 1 U S P Unit
	= 1 M R C Unit
	= 1 Coward Unit
	= 0.025 γ Vitamin D ₂
	= 5-6 Poulsson Units
	= 1.66 Oslo Units
	= 6-8 Laquer Units
	= 2.6 Trophylactic Units
	= 3.25 ADMA Units
1 Clinical Unit	= 12.5-17 I U
1 Steenbock Unit	= 3.2 I U
1 Standard Unit	= 1,000,000 I U

24 Metabolism

Vitamin D can be successfully administered in various ways for example by oral ingestion, by parenteral injections and by absorption through the skin.³⁷⁰ (See page 410 for the reported quantitative differences in the efficacy of the vitamins D₂ and D₃ when administered intramuscularly or *per os*.) The environmental temperature seems to be an additional determining factor in the response of the organism to vitamin D.³⁷¹

³⁶⁹ Assoc. Official Agr. Chem. *Methods of Analysis*, Fifth Ed. 1940, p. 371.

³⁷⁰ L. Krenn, *Munch. Med. Wochschr.* 86: 1317 (1939); E. M. Hume, N. S. Lucas and H. H. Smith, *Biochem. J.* 21: 362 (1927).

³⁷¹ D. T. Riellotte and W. L. Bacon, *J. Nutrition* 10: 683 (1933).

Vitamin D is absorbed from the intestines, especially in the small gut. The absorption is facilitated by the presence of fat but excess doses of fat give less favorable results.³⁷² Vitamin D is not absorbed from mineral oil.³⁷³ Effective absorption of vitamin D is related to the presence of bile.³⁷⁴ Thus, animals with biliary fistula cannot utilize vitamin D when given by mouth unless administered simultaneously with a bile acid, such as taurocholic acid or desoxy-cholic acid.³⁷⁵ Vitamin D is not well absorbed in obstructive jaundice. Vitamin D esters of acids which can be hydrolyzed in the intestines are antirachitically effective while esters which cannot be hydrolyzed also cannot be utilized.³⁷⁶

From the intestines vitamin D is absorbed into the blood³⁷⁷ and distributed all over the organism. The healthy body contains definite amounts in the lymph and tissue fluids. Normal human blood contains from about 50 to 135 International Units per 100 cc of serum (average about 100) while rabbits were found to have a mean of approximately 50.³⁷⁸ The human body and also all animals investigated, with the exception of fish, have no special storage organ for this vitamin, although substantial amounts can be found³⁷⁹ in various organs such as lung, liver, spleen, brain and wherever fat is accumulated as long as there is no shortage of this vitamin in the organism. The heart has consistently been found to be devoid of any stored amounts of this vitamin. The liver and to a certain extent, also the viscera are special storage organs only for fish.

Vitamin D is readily metabolized as is evident from the transfer of ingested vitamin D into milk of all mammals and into the eggs of birds. The type of vitamin D fed is also secreted. Thus, vitamin D₂ or D₃ is found in milk³⁸⁰ or in eggs^{391, 392} according to which form has been fed to the animal. There is apparently no principal difference in the utilization of the various forms of vitamins D since vitamin D from fish liver oils³⁹³

³⁷² A. Knudson and R. J. Floody *J. Nutrition* 20 317 (1940)

³⁷³ M. C. Smith and H. Spector *Proc. Am. Soc. Biol. Chem.* 134 XC (1940) *J. Nutrition* 20 1 (1940)

³⁷⁴ W. Heymann *J. Biol. Chem.* 122 249 (1937)

³⁷⁵ J. D. Greaves and C. L. A. Schmidt *Ibid.* 102 101 (1933) *Univ. Calif. Pub. Physiol.* 8 49 (1934)

³⁷⁶ A. Windaus and O. Rygh *Nachr. Ges. Wiss. Göttingen Math. physik. Klasse* III 202 (1928)

³⁷⁷ A. F. Hess, R. F. Light, C. N. Frey and J. Gross *J. Biol. Chem.* 97 369 (1932) A. F. Hess, M. Weinstock and J. Gross *Proc. Soc. Exptl. Biol. Med.* 30 1357 (1933)

³⁷⁸ J. Warkany *Z. Kinderheilk.* 49 191 259 (1930) *Am. J. Diseases Children* 49 318 (1933) 52 831 (1936) *Biochem. Z.* 293 415 (1937)

³⁷⁹ H. Goldblatt and K. M. Soames *Biochem. J.* 17 446 (1923) I. H. Page *Biochem. Z.* 220 420 (1930) W. Heymann *J. Biol. Chem.* 118 371 (1937)

³⁸⁰ R. M. Bethke, W. E. Krauss, P. R. Record and O. H. M. Wilder *J. Nutrition* 11 21 (1936)

³⁹¹ R. M. Bethke, P. R. Record, C. H. Kirk and D. C. Kennard *Poultry Sci.* 15 326 (1936)

³⁹² R. M. Bethke, P. R. Record, O. H. M. Wilder and C. H. Kirk *Ibid.* 15 336 (1936)

³⁹³ For example R. M. Bethke, D. C. Kennard and H. L. Sassaman *J. Biol. Chem.* 72 695 (1927)

from activated ergosterol,³⁸⁴ from activated 7 dehydro cholesterol and activated "mussel provitamin"³⁸⁵ are all metabolized as described, although a small difference in the efficiency of this process for different vitamins D has been observed, that is, vitamin D₂ is somewhat less effectively utilized than the other forms of vitamin D.^{385 386 387} Colostrum (of cows³⁸⁸) contains from six to ten times the amount of vitamin D found in normal milk. Also of special interest is the fact that vitamin D apparently can pass only in limited but definite amounts through the placental walls. New born animals and babies have practically no vitamin D in their tissues³⁸⁹ even though their mothers had an abundant supply. On the other hand when the mother's diet was deficient, the bones and skull of the infant were found softer than normal and the teeth when erupted showed defective formation.³⁹⁰ All these observations suggest that a special regulatory mechanism exists which takes care of the vitamin D requirements of the embryo.

Some of the vitamin D is destroyed in the organism, some is excreted.³⁹¹ No quantitative balance studies are available which would indicate to what extent destruction occurs. Excretion occurs only through the intestinal tract and mainly through the bile but not through the kidneys. The extent to which vitamin D is secreted through the skin is unknown.

25 Physiological Action

An enormous amount of work has been done in various studies to define the action and the mode of action of vitamin D. The result of all these investigations has not yet developed into a well rounded picture. Most work has been done either with vitamin D₂ or with fish liver oils and it seems quite certain that at least some differences may be found in the specific reactions of these forms of vitamin D and of vitamin D₃ which is considered to be the natural vitamin D of man and higher animals.

³⁸⁴ R. F. Light, L. T. Wilson and C. N. Frey, *J. Nutrition* 8, 105 (1934).

³⁸⁵ J. van N. ekerk and Hofstra, *Tijdsch. Diergeneesk.* 1939, 60.

³⁸⁶ G. M. Delaney, H. E. Mansell and H. W. Tuttle, *Pol. y Sci.* 12, 10 (1933).

³⁸⁷ R. M. Bethke, P. R. Record, O. H. M. W. lder and C. H. Kirk, *Ibid.* 15, 336 (1936).

³⁸⁸ J. van N. ekerk and M. S. C. Bliek, *Acta Brera V. la d. Physiol. Pharmacol. Microbiol.* 9, 20 (1939).

³⁸⁹ A. F. Hess and M. Weinstock, *Am. J. Diseases Children* 27, 1 (1924); *J. Am. Med. Assoc.* 83, 15, 8 (1924); C. E. Bills, *J. Biol. Chem.* 72, 751 (1927); C. E. Bills and A. M. Wirrick, *Ibid.* 86, 117 (1930).

³⁹⁰ K. W. Tord and G. Toverud, *Acta Paediatr.* (suppl. 2) 12, 1 (1936).

³⁹¹ A. F. Hess, R. F. Light, C. N. Frey and J. Gross, *J. Biol. Chem.* 97, 369 (1932); A. F. Hess, M. Weinstock and J. Gross, *Proc. Soc. Exptl. Biol. Med.* 30, 13, 7 (1933).

In the broadest aspect vitamin D stimulates growth³⁹² While this property is apparently common to all vitamins at least as a secondary reaction, it seems of primary significance in the case of vitamin D since growth of all animals stops or is retarded in the absence of this vitamin On the basis of this property a method for the determination of vitamin D has been proposed³⁹³ This retardation of growth is probably quite a fundamental metabolic process Thus eggs which contain an insufficient amount of vitamin D or which have been laid by hens that obtained suboptimal doses of this vitamin do not hatch³⁹⁴

The growth of bones can easily be demonstrated to be related to the action of vitamin D although the possibility should not be overlooked that the growth of cells other than bone cells may also be influenced

The physiological study of the action of vitamin D must necessarily commence with a study of the organism deprived of this vitamin and proceed to a determination of the effects brought about by the action of the vitamin and finally end with an explanation of the vitamin action

Whereas the clinical symptoms of a vitamin D deficiency will be discussed in the section on avitaminosis the broad picture of the disease and the metabolic changes must be mentioned here The clinical symptoms of the vitamin D deficiency are commonly known under the term rickets and the gross effect is obviously a disturbance of the mineral metabolism Actually the deposition of the inorganic calcium phosphorus salts in the bones is retarded or stopped entirely thus retarding or stopping the growth of bones Furthermore the inorganic material previously deposited may be withdrawn from the bones causing a considerable softening An early symptom of a vitamin D deficiency is a lowered content of phosphorus in the blood serum and later also a lowering of the calcium level is observed³⁹⁵ ³⁹⁶ Furthermore a general decreased retention of phosphorus and at later stages of the deficiency also a decreased retention of calcium are found Thus the phosphorus metabolism is much more hampered than the calcium metabolism All these changes are not necessarily the primary ones but represent the ultimate effect of the vitamin deficiency which can be observed

To study the physiology and the mode of action of vitamin D animal experiments must be performed Experimental rickets was first obtained

³⁹² G Stearns P C Jeans and V Vandecar *J Ped at* 9 1 (1931) F Slyker B M Hamil M W Poole T B Cooley and I G Macy *Proc Soc Exptl Biol Med* 37 499 (1937)

³⁹³ K H Coward K M Key and G E Morgan *Biochem J* 26 1085 (1932)

³⁹⁴ J S Carver I I Robert in D Hazen R H Johnson and J I St John *Washington Ter Exptl Station B II* 299 (1934)

³⁹⁵ J Howland and B Kramer *Am J Diseases Children* 22 103 (1971)

³⁹⁶ P Iversen and E Istrup *Forhandl Forsk nordt Læge Paed afr (Copenhagen)* 1920 83

in dogs³⁸⁷ but soon rats were used^{393 399} and most of the work has been done with these animals. Rats however, have usually a very low requirement for vitamin D. That is, on a vitamin D deficient, but otherwise normal diet they do not develop classical rickets. A condition, however, which resembles human rickets can be brought about by special rations which have a disproportion between calcium and phosphorus. Thus a diet of high calcium and low phosphorus content or of low calcium and high phosphorus content produces experimental rickets in rats. In addition to the ratio of calcium to phosphorus the absolute amount of each also determines the rachitogenic properties of the diet.⁴⁰⁰ Very satisfactory results were obtained with the diets Steenbock and Black No 2965⁴⁰¹ and with McCollum No 3143⁴⁰² which are similar in composition and which contain about 1.2% of calcium and about 0.25% of phosphorus the ratio of calcium to phosphorus thus being about 5:1. Rats reared on such rations develop dietary rickets that can be cured by the administration of vitamin D.

Physiological studies on rats have given rise to a number of theories on the action of vitamin D. None of these theories however should be generalized until confirmatory evidence from experiments with other more suitable species has been obtained. In contradistinction the mode of action of vitamin D should be investigated in animals which develop rickets only by a deficiency of vitamin D and in which rickets cannot be prevented by a regulation of the calcium and phosphorus content of the diet. Thus dogs⁴⁰³ pigs⁴⁰⁴ and many birds⁴⁰⁵ especially the hen and the turkey, are useful experimental animals.

The histological changes that occur during bone formation must be discussed briefly in order to understand the action of vitamin D. There are three different types of tissue concerned with the growth of bones—the cartilage tissue the osteoblasts and the bone tissue. During the course of normal bone growth a certain number of cartilage cells de-

³⁸⁷ E. Mellanby *J. Physiol.* 52 LIII (1919). *J. ucel* 1 407 (1919).

³⁸⁸ H. C. Sherman and A. M. Pappenheimer *Proc. Soc. Exptl. Biol. Med.* 18, 193 (19 0/1921). *J. Exptl. Med.* 34 189 (1921).

³⁸⁹ I. V. McCollum and N. Simmonds *J. Biol. Chem.* 47 111 139 175 207 235 507 (1921). See also V. Korenchvsky *B. i. Med. J. No.* 3171 547 (1921). *Special Rept. Sci. Med. Resear. Ch. Council* No 71 (19 2).

³⁹⁰ H. B. Brown, A. T. Shohl, H. E. Chapman, C. S. Rose and E. M. Bauerweil *J. Biol. Chem.* 98 207 (1932). A. Querdo *Arch. neerland. physiol.* 20 487 (1935). A. T. Shohl and C. B. Wolfbach *J. Nutrition* 11 275 (1936).

³⁹¹ H. Steenbock and A. Black *J. Biol. Chem.* 64 63 (19 5).

³⁹² F. V. McCollum, N. Simmonds, P. C. Shopley and E. A. Parks *Ibid.* 51 41 (1922). 47 30 (1921). *Proc. Soc. Exptl. Biol. Med.* 18 275 (19 1). *Am. J. Hyg.* 1 492 (1921).

³⁹³ E. Mellanby *J. Physiol.* 52 LIII (1919). *Lancet* 1 407 (1919).

³⁹⁴ Schoch *Mitt. Lebensm. Hyg.* No. 1/2 176 (1933).

³⁹⁵ O. Wambier *Antirachitosis D. hos Kyllis ge* (Thesis) Copenhagen 1939.

While only the relation of vitamin D to the calcium and phosphorus metabolism has been discussed, there is evidence that vitamin D is also concerned with a number of other reactions, either directly or indirectly. For example, vitamin D has an influence upon the metabolism of other minerals, especially of magnesium and iron. Vitamin D, furthermore, affects the carbohydrate metabolism, since during vitamin D avitaminosis the phosphorylation of carbohydrates is retarded.⁴³⁰ Vitamin D, or ultra violet light, leads to changes in carbohydrate metabolism (experiments with rats) which are very similar to those observed by administration of insulin. An increase in the glycogen in the liver, and to a lesser degree in the muscle, has been observed. Furthermore, the quotient carbohydrate/lactic acid goes up in blood, liver and muscle.⁴³¹ A relation of vitamin D to the fat metabolism has also been postulated.

26 Relation to Other Vitamins and Hormones

As has been postulated in the general chapter on the interrelationship of vitamins and on the relationship of vitamins to hormones (pages 29 to 31), no single vitamin is able to exert its full action in the absence of other necessary substances. The effects which occur when only vitamin D is taken out of the vitamin balance have been discussed. A multiple deficiency of vitamin D together with another or several other vitamins has not been observed other than in the form of added symptoms from each deficiency. It has been claimed that the effect of toxic amounts of vitamins D can be relieved by simultaneous administration of the vitamins of the B group such as are present in yeast.⁴³²

At various times relations of vitamin D to different glands or hormones have been postulated. The thyroid gland and the parathyroid gland have been the center of discussion. It has been claimed that the administration of thyroid extracts cures rickets,⁴³³ but it has also been claimed that thyroid intensifies the symptoms of rickets. The parathyroid gland influences the calcium metabolism, but in an entirely different manner from the influence that vitamin D exerts. Parathyroid raises primarily the calcium level in blood serum. Vitamin D, however, raises the phos

⁴³⁰ E. Freudenberg and A. Welker *Z. Kinderheilk.* 41: 466 (1926). H. Hentschel and E. Zöller *Ibid.* 44: 146 (1927).

⁴³¹ L. Pincussen *Proc. Am. Soc. Biol. Chem.* 1941: CI.

⁴³² L. J. Harris and T. Moore *Biochem. J.* 22: 1461 (1928). *Lancet* I: 892 (1928). R. F. Light, G. Miller and C. N. Frey *J. Biol. Chem.* 84: 287 (1929). E. R. Norris and A. E. Church *Ibid.* 89: 437 (1929). H. J. Jusatz *Z. ges. exp. Med.* 87: 529 (1933). *Z. Vitaminforsch.* 3: 268 (1934). *Klin. Wochschr.* II: 1501 (1932). Herrmann *Klin. Wochschr.* II: 1752 (1929).

⁴³³ A. Nitschke *Z. ges. exp. Med.* 8: 236 (1932). *Z. Kinderheilk.* 54: 233 (1933). *Klin. Wochschr.* 12: 1793 (1933).

phorus level Parathyroid causes an increase of ionized calcium in blood, vitamin D of bound calcium Parathyroid stimulates the withdrawal of calcium from the body, vitamin D stimulates its retention All these facts caused various investigators to find either a synergistic or an antagonistic effect of parathyroid on rickets according to the type of experiments carried out It now seems that the action of both compounds is independent

The secretions of various other glands for example the anterior pituitary, have been claimed to be interrelated with the action of vitamin D Thus the secretions of the lymph glands are said to influence the phosphorus and the calcium content of serum ⁴³⁴ It has been suggested that the ovaries influence rickets, since mothers after the parturition sometimes become rachitic ⁴³⁵ Finally the thymus has been reported to be involved in the functioning of vitamin D ⁴³⁶ All these findings and theories need further study since there is no agreement, as yet, about the relation between these effects and vitamin D

27 Hypovitaminosis and Avitaminosis

The clinical symptoms of vitamin D deficiency in infants and in young animals are called rickets This disease occurs most frequently in man beginning around the fourth month of age but also occurs in children of school age Early signs of the disease are noticeable continuous discomfort and perspiration on the head Soon the typical skeletal changes especially in the ribs forearm and wrist become apparent and can be recognized by roentgenograms The retardation in ossification of the fontanelles is especially characteristic in babies ⁴³⁷ The change in the bones is a lack of calcification which becomes especially noticeable at the epiphysis As a result, the ends of the long bones become greatly enlarged by excessive cartilage formation Also enlargements of the junctions between the bones especially between the ribs and the cartilages which are normally present (rachitic rosary) occur In more serious cases the skull is malformed The jaw may be ill shaped the teeth appear late grow too close to each other and possess ill formed enamel ⁴³⁸ Deficiency of vita

⁴³⁴ A Nitschke *Z ges expil Med* 65 637 651 (1909) *Dent Med Wochschr* 62 629 (1936)

⁴³⁵ Hansen *Korrespbl Schweiz Ärzte* 22 497 (1892)

⁴³⁶ G Lucander *Boll soc ital biol sper* 13 8 (1938) *O Hrota Folia Endocinol Japon* 13 46 (1937)

⁴³⁷ Farber E J Dalyell and Mackay *Med Research Council Rept on Rickets in Vienna Spec Rept No 77* (1937)

⁴³⁸ M M Eliot E P Southern B A Anderson and S Armin *Am J Diseases Child en* 46 453 (1933)

min D is to a certain extent, but not altogether, responsible for the occurrence of dental caries⁴³⁹ The elastic properties of the bones are generally disturbed as evidenced, for example, in rat bones by breaking load and deflection stress⁴⁴⁰ A curvature of all bones occurs in later stages of the disease, and is especially significant in the limbs the spine, etc

A vitamin D deficiency, however, does not affect the bones exclusively but the entire body Besides the changes noted on the bones, the effects on muscles are most obvious During severe cases of rickets the muscles become weak and flabby

During vitamin D deficiency the organism is particularly susceptible to a number of infectious diseases which sometimes cause death such as bronchopneumonia, tuberculosis, infectious fevers etc It has also been reported⁴⁴¹ that milk fever can be prevented in cows by a vitamin D supplement fed prior to parturition

A special symptom is spasmophilia (infantile tetany)¹ As previously discussed, this disease when caused by a deficiency of vitamin D is considered to indicate the beginning of healing In typical rickets, tetanic spasms often occur of either a general nature or localized in the hands and feet Sometimes cramps occur even in the heart muscles and the bronchial muscles Rickets of the adult is also called 'osteomalacia' and occurs especially in women during and after pregnancy but has also been noted sporadically among men and women of all ages (osteoporosis) The general symptoms are exactly the same as those of the baby rickets namely, de calcification of the bones leading to brittleness

(a) Clinical Test Methods

1 X-Ray Determination This method is the oldest and the one that is most commonly used but reveals only cases of avitaminosis, whereas the state of hypovitaminosis cannot be detected The roentgenographic examination of the bones of the forearm and the wrist is especially recommended both for diagnosis of rickets and for following the healing process

2 Determination of Blood Ca and P The normal level is 4-6 mg % of P and 9-11 mg % of calcium Any value below these is considered to indicate vitamin D deficiency The determination of Ca and of P is

⁴³⁹ M. Mellanby and J. D. King *Ergeb. Vitamins Hormonforsch.* 2: 1 (1939) C. F. Taylor and C. D. M. Day *Brit. Med. J.* 1: 919 (1939)

⁴⁴⁰ A. A. Schiller, H. C. Struck and C. I. Reed *Proc. Am. Physiol. Soc.* 1941: 250

⁴⁴¹ J. R. Grieg *Scottish J. Agr.* 13: 369 (1930) B. Sjollema *Nutrition Abstracts & Revs.* 1: 671 (1932) F. H. Conover *Vet. Med.* 35: 657 (1940) T. M. Olson *South Dakota Exptl. Station Bull.* 319: 1938

carried out according to standardized procedures⁴⁴² The product of the values found for Ca and P is also used as criterion and should be above 30 in normal individuals This evaluation procedure is considered to give trustworthy results only in cases of severe avitaminosis and cannot be used to follow the progress during treatment

3 Blood Phosphatase Test⁴⁴³ This test is based on the fact that the enzyme phosphatase occurs in increasing amounts in the blood during bone diseases such as rickets When rickets is healed, the amount of phosphatase in blood is again slowly but not immediately reduced

4 Mineral Metabolism Test⁴⁴⁴ This test in which the phosphorus⁴⁴⁵ and calcium⁴⁴⁶ balance is determined is considered, in the hands of experts, to give the best data as to the state of rickets In early disease much more phosphorus is excreted than calcium At later stages calcium and phosphorus are both excreted in increased amounts The beginning of healing is characterized by a marked phosphorus retention and when healing is well under way a considerable retention of calcium and phosphorus is observed

28 Hypervitaminosis

Vitamins D given in large excess to any experimental animal or man are toxic It is therefore important to know the symptoms of such a D hypervitaminosis and the minimum amount of vitamin D which may cause an intoxication

The first sign of a D hypervitaminosis is digestive disorder There is a loss of appetite, vomiting and diarrhea A considerable loss of weight, an inflammation of the kidneys and finally death occur Excessive doses of vitamin D cause an increase of the calcium content of the serum which may reach a value of 17 mg % As a result metastatic calcification occurs⁴⁴⁷ in various organs and tissues especially in the kidneys stomach lungs heart blood vessels and bronchi At first a retention of calcium in the organism is observed but at later stages a decalcification of the skeletal

⁴⁴² For example F Müller *Z Kinderheilk* 57 243 (1935) *Z physiol Chem* 237 35 (1935) See also L Pincussen *Arbeitsmethoden* Leipzig 1930

⁴⁴³ H D Kay *J Biol Chem* 89 325 (1930) *Physiol Rev* 12 384 (1932) N Morris and O D Peden *Quart J Med* 6 211 (1937) *Arch Disease Childhood* 12 45 (1937) D J Barnes and A D Carpenter *J Pediatr* 10 96 (1937)

⁴⁴⁴ E Rominger et al *Arch Kinderheilk* 80 195 (1917) 81 176 (1917) E Rominger H Meyer and C Bomskov *Acta Med Scand* 11 1391 (1930) 11 1293 1342 (1931) *Z ges exper Med* 73 341 (1930) 78 259 272 (1931)

⁴⁴⁵ Phosphorus determination for example according to E Müller *Z physiol Chem* 237 35 (1935) *Z Kinderheilk* 57 243 (1935)

⁴⁴⁶ Calcium determination for example according to B Kramar and F F Tisdall see L Pincussen *Arbeitsmethoden* Leipzig 1930

bones sets in. Finally, phosphorus is excreted and calcium deposited in the tissues. Shortly before death an excretion of calcium through the kidneys also occurs.⁴⁴⁷

Unfortunately, no exact data for the toxic dose can be given. This is due mainly to the fact that the toxic threshold varies considerably among individuals. As an average figure a continued daily dose of about 20,000 International Units of vitamin D per kilogram of body weight may be considered to cause intoxication in man and dogs.

Clinically, in the so called shock therapy, single doses up to 1 000,000 International Units of pure crystallized vitamins D dissolved in a suitable medium have been used and no injuries have been observed from such treatments.

The over all toxic effect of vitamin D₂ is believed to be somewhat greater than that of vitamin D₃ (studied on dogs). After feeding dogs excessive amounts of these two forms of vitamin D the animals were allowed to recover. Functional recovery was rapid in the dog relieved from vitamin D₃ but the damage to tissues was more severe and less repaired than in the animal relieved from vitamin D₂.⁴⁴⁸

It should, furthermore, be noted that toxic symptoms have been observed only when vitamin D was given *per os* or parenterally, but never when vitamin D was supplied by ultraviolet irradiation. The deleterious effect observed on the normal organism upon over irradiation is independent of vitaminization. This suggests that a special protective mechanism exists in the body probably in the skin which takes care of the potential effects of an over irradiation of the provitamin D.

29 Requirements

The human requirements of vitamin D⁴⁴⁹ are difficult to estimate correctly due to an individual variation in the utilization of dietary calcium and phosphorus without added vitamin D.⁴⁵⁰ The optimum amount of vitamin D for babies, children and adolescents is believed to be about 400 to 800 International Units per day provided a sufficient amount of calcium and phosphorus is offered. The best combination of the necessary minerals with vitamin D is found in milk, which may be fortified with vitamin D. The need of adults for vitamin D appears to be somewhat smaller but exact data are not available. Pregnant and lactating women⁴⁵¹

⁴⁴⁷ C. A. Ashford *Biochem. J.* 24: 661 (1930). L. I. Harris and J. R. M. Innes *Ibid.* 25: 367 (1931).

⁴⁴⁸ A. F. Morgan, J. B. Hendricks and R. M. Freytag *Proc. Am. Soc. Biol. Chem.* 1941: XCII.

⁴⁴⁹ P. C. Jeans and G. Stearns *J. Am. Med. Assoc.* 111, 703 (1938).

⁴⁵⁰ H. A. Hunscher, F. C. Hummel and S. G. Macy *Proc. Soc. Exptl. Biol. Med.* 35: 189 (1936).

⁴⁵¹ K. W. Toverud and G. Toverud *Acta Paediat.* (Suppl. 2) 12: 1 (1936).

are advised to take at least 800 I U per day Babies born prematurely and twins need increased amounts (See also page 613 for the recommended daily allowances as established by the National Research Council)

These requirements as stated pertain only to the ingested forms of vitamin D and to the optimum daily intake of normal organisms for protection against rickets To cure rickets in infants a daily dose of from 500 to 1500 International Units is usually given The method of supplying the body only once every three to six months instead of every day is applied in the so called shock therapy Massive doses of 200 000 to 1 000 000 International Units have been recommended and used for this purpose ⁴⁵ ⁴⁵³ ⁴⁵⁴

The vitamin D requirement of poultry has been studied extensively because of its practical importance and is usually expressed in vitamin D content per pound of feed It has been recommended to incorporate about 180 A O A C chick units of vitamin D per pound of total feed for growing chicks, while for the laying stock 360 A O A C chick units and for the breeding stock 540 A O A C units are required

The vitamin D requirement of species other than man and poultry has not been investigated quantitatively Some data have been presented which indicate that dogs especially dogs of large breeds such as Great Dane, Setter Airdale and German Shepherd need considerably more vitamin D than small breeds such as Terrier and Spaniel ⁴⁵⁵ In terms of vitamin D₂ small breeds need about 28 U S Pharmacopoeia Units per kg of body weight while large breeds need ten times this amount or even more It seems conceivable that this species difference is due to the compound specificity of the vitamin D₂ used and that the vitamin D requirement of dogs is more uniform and considerably lower when other forms of vitamin D such as vitamin D₃ are fed In dogs a greater tendency toward rickets has been observed in males than in females ⁴⁵⁶

Swine and especially young pigs need vitamin D and when reared on a low vitamin D intake became definitely rachitic especially in the winter months ⁴⁵⁷ Vitamin D given to pigs resulted also in a considerable decrease in the amount of feed necessary for maximum growth ⁴⁵⁸ A daily ad

⁴⁵³ S Gunnarsson *Acta Paediat* 25 89 (1939)

⁴⁵⁴ J Ström *Ibid* 25 251 (1939)

⁴⁵⁵ G O Harnapp *Klin Wochschr* 15 1043 (1936) *Monatsschr K d rthik* 71 193 (1937) *Klin Wochschr* 17 390 (1938)

⁴⁵⁶ A F Morgan *North Am Vet* 21 462 (1940)

⁴⁵⁷ C R Stockard *Am J Dis Assoc Children* 36 310 (19 8)

⁴⁵⁸ G Bohstedt R M Bethke B H Edgerton and W L Robinson *Ohio Exptl Station Bull* 395 (1926) W C Skelley *J Exptl Stat on B* 11 661 (1939)

⁴⁵⁹ R D Sinclair *Sci Ag* 9 629 (1939)

ministration of approximately 110 U S Pharmacopoeia Units vitamin D₂ has been suggested for pigs per kg of body weight. Sheep also benefit from an intake of vitamin D⁴⁵⁹ and many reports have been published showing the need of cattle and especially of calves for vitamin D⁴⁶⁰. A daily intake of about 200 U S Pharmacopoeia Units vitamin D per kg of body weight has been suggested for these animals. For horses 200 to 2000 U S Pharmacopoeia Units have been given per kg of body weight for the cure of rickets⁴⁶¹.

⁴⁵⁹ D W Auchinachie and A H H Fraser *J Agr Sci* 22 560 (1932)

⁴⁶⁰ I W Rupel G Bohstedt and E B Hart *Wisconsin Expt Station Research Bull* 115 (1933) N W Hilston (Thesis) The Pennsylvania State College 1937 T W Gulliken L S Palmer and W L Boyd *Minnesota Agr Stat Techn Bull* 105 (1935)

⁴⁶¹ J H Kinter and R L Holt *Philippine J Sci* 49 1 (1932)

**THE GROUP OF
VITAMINS E**

THE GROUP OF VITAMINS E

The physiological effect of vitamin E is brought about by a series of naturally occurring compounds, which are chemically very closely related being homologs and isomers of each other. Three different compounds have been isolated which are designated as α , β and γ tocopherol. The possibility that other vitamin E factors may occur in the animal or plant organism cannot be excluded but no definite proof for the existence of other compounds can be offered.

1 Nomenclature and Survey

Names

Vitamin E^{1, 2}

Tocopherols³ (tokos (Greek) meaning childbirth phero (Greek) meaning to bear)

Anti encephalomalacia vitamin

Factor X⁴

Antisterility factor

Reproductive vitamin

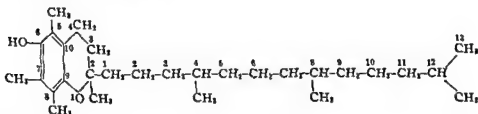
Sterilamine⁵

Fertility vitamin.

The members of the group of vitamins E

1 α Tocopherol $C_{29}H_{50}O_2$

Synonym 5,7,8 Trimethyl tocol⁶



¹ H. M. Evans and K. S. Bishop *Science* 56: 650 (1921) *Am. J. Physiol.* 63: 296 (1922) *J. Am. Med. Assoc.* 81: 889 (1922) *J. Metabolic Research* 1: 319, 335 (1922)

² B. S. re *J. Biol. Chem.* 59: 19 (1944)

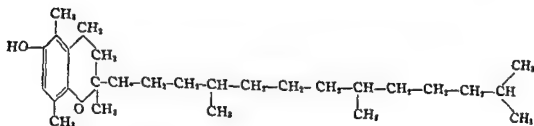
³ H. M. Evans, O. H. Emerson and G. A. Emerson *Ibid.* 113: 319 (1936)

R. L. Jones *Science* 63: 480 (1948)

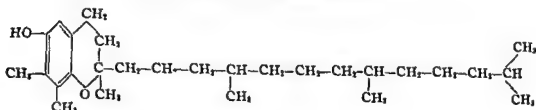
P. Karrer and H. Fritzsche *Helv. Chim. Acta* 21: 1234 (1938) proposed the term tocol for the general class of tocopherols without substituents in the benzenoid nucleus. The nomenclature is indicated by following the Geneva nomenclature principle for chroman. Accordingly α tocopherol is 5,7,8-trimethyl tocol β tocopherol is 8-dimethyl tocol etc.

2 β Tocopherol $C_{55}H_{100}O_2$

Synonyms Cumo tocopherol⁸ neo tocopherol⁹ *p*-xylo tocopherol⁷ 5,8-dimethyl tocol⁸

3 γ Tocopherol $C_{55}H_{100}O_2$

Synonyms *o*-Xylo tocopherol⁷ 7,8-dimethyl tocol⁸



Efficacy

1 g α tocopherol = 400 Rat Units

1 g β tocopherol = 200 Rat Units

1 g γ tocopherol = 200 Rat Units

1 g synthetic racemic tocopherol acetate (*dl* α tocopherol acetate) = 1000 International units

2 Chronology

1920 MATILL and CONKLIN⁸ observed disturbances in the reproduction of rats on special milk diets

1922 EVANS and BISHOP⁹ reported failure of rats to reproduce when on a purified diet and recognized the missing factor as a vitamin

1927 EVANS and BURR¹⁰ recognized that vitamin E is non saponifiable

1928-1930 EVANS and BURR¹¹ and GOETTSCHE and PAPPENHEIMER¹² described the

⁸ W. John Z. *physiol Chem* 250 11 (1937)

⁹ According to a suggestion by O. H. Emerson and L. I. Smith *J Am Chem Soc* 62 1869 (1940) the substitution in the aromatic ring of the tocopherol structure is indicated in the name of the tocopherol by a prefix which describes the substitution in terms of simple benzene derivatives such as *o*, *m* and *p*-xylo-tocopherol, tolu tocopherols, etc.

¹⁰ H. A. Matill and R. E. Conklin *J Biol Chem* 44 137 (1920)

¹¹ H. M. Evans and K. S. Bishop *Science* 56 650 (1922) *Am J Physiol* 63 396 (1922) *J Am Med Assoc* 81 889 (1922) *J Metabolic Research* 1 319 335 (1922)

¹² H. M. Evans and G. O. Burr *Mem Univ Calif* No 8 (1927)

¹³ H. M. Evans and G. O. Burr *J Biol Chem* 75 273 (1928)

¹⁴ M. Goettsch *Proc Soc Exptl Biol Med* 27 584 (1930) A. M. Pappenheimer *Ibid* 27 567 568 (1930) M. Goettsch and A. M. Pappenheimer *J Exptl Med* 54 145 (1931)

occurrence of a specific muscular dystrophy in rats rabbits and guinea pigs on a vitamin E-deficient diet

- 1936 EVANS EMERSON and EMERSON¹² isolated two different vitamins E (α and β tocopherol) in the form of crystallized esters
- 1937-1938 FERNHOLZ¹³ elucidated the chemical structure of α tocopherol
- 1938 KARRER followed by I I SMITH and by TODD synthesized tocopherol¹⁴

3 Occurrence

The group of vitamins E occurs predominantly in plant materials The animal organism contains only small amounts

The best natural source of vitamins E is vegetable oils such as wheat germ oil which contains especially high amounts Varying amounts of this group of vitamins are found in cottonseed oil¹⁷ rice germ oil and other seed germ oils¹⁸ Olive oil does not contain any vitamins E arachis oil contains traces¹⁹ Lettuce alfalfa etc contain considerable amounts oranges and bananas small amounts Animal materials contain little vitamin E The highest amount has been found²⁰ in livers (of horse and cattle but not of rats²¹) and small amounts are present in the muscles heart kidneys placenta milk and eggs Fish liver oils which are especially rich in the vitamins A and D are poor in vitamin E

Different vitamins E or different mixtures of vitamins E occur in the various natural sources Thus α and β tocopherols have been found in wheat germ oils but not always in the same relative proportions In European oil the β compound is the main principle while in American sources the α form is the predominant factor with smaller quantities of γ tocopherol²³ Cottonseed oil palm oil and corn oil²⁴ contain predominantly γ tocopherol besides small amounts of α tocopherol

The tocopherols occur in the free form²⁵ at least to a considerable extent in the seed oils It is believed that some occur esterified but no definite data concerning this are available

¹² H M Evans O H Emerson and G A Emerson *J Biol Chem* 113 310 (1936)

¹³ F Fernholz *J Am Chem Soc* 59 1184 (1937)

¹⁴ F Fernholz *Ibid* 60 700 (1938)

See the literature references on p 44

¹⁵ H S Olcott *J Biol Chem* 107 471 (1934)

¹⁶ H M Evans and G O Burr *Proc Natl Acad Sci U S A* 11 334 (1925) H S Olcott and H A Matill *J Biol Chem* 104 423 (1934)

¹⁷ A L Bacharach E Alleborne and H E Glynn *Biochem J* 31 2287 (1937)

¹⁸ P Karrer W Jaeger and H Keller *Helv Chim Acta* 23 464 (1940)

¹⁹ W F J Cuthbertson R R Ridgeway and J C Drummond *Biochem J* 34 34 (1940)

²⁰ T Moore A J P Martin and K R Rajagopal *Soc Chem Ind Fed G O p* 1939 41

²¹ A R Todd F Bergel and T S Wok *Biochem J* 31 7237 (1937)

²² O H Emerson G A Emerson and H M Evans *Science* 89 193 (1939)

²³ A R Moss and J C Drummond *Biochem J* 32 1953 (1938)

The tocopherols occur naturally together with other compounds of unknown constitution which are even stronger antioxidants than the vitamins E and which protect the vitamins against oxidation

4 Isolation

The isolation of the vitamins E is usually carried out by first isolating the unsaponifiable part of the vitamin E containing material. Wheat germs for example are dried and extracted with an organic solvent, such as chloroform, ether, etc. Another method is to press the germs, to collect the oil and to extract the residues. The total fats are then saponified at room temperature with, for example, 20% alcoholic potassium hydroxide in the absence of oxygen and the non saponifiable part (approximately 5% of the oil) is extracted with an organic solvent. Sterols constitute up to 90% of the non saponifiable material and are separated by crystallization from suitable solvents such as alcohols, pentane, etc. The last traces are then removed by precipitation with digitonin. The remaining oil can be purified by distillation, the vitamin E containing fraction being carried over at 200–250° C at 0.1 mm pressure^{26 27 28}. Some of the vitamin is, however, lost in this procedure. Some purification of the unsaponifiable mass can be achieved by partition between different solvents, such as methanol and petroleum ether,^{29 30} whereby the vitamin goes into the latter. By utilization of the principle of the chromatographic adsorption on aluminum oxide, a certain further purification can be achieved^{31 32}.

Instead of first isolating the non saponifiable part the vitamins E can also be obtained in improved yields directly from, for example, wheat germ oils, by application of the principle of the chromatographic adsorption method³³.

Final isolation of the vitamins E is achieved by precipitation in the form of a crystallized ester. The allophanates are especially useful for this purpose³⁴. By fractional crystallization of these esters, the members of the group of vitamins E are separated. The most insoluble fraction represents the α tocopherol and from its mother liquors the allophanates of the β

²⁶ H S Olcott *J Biol Chem* 107 471 (1934)

²⁷ H S Olcott and H A Matill *Ibid* 104 473 (1934) H S Olcott *Ibid* 110 695 (1935)

²⁸ F W Quackenbush, H L Gottlieb and H Steenbock *Ind Eng Chem* 33 1276 (1941)

²⁹ H M Evans, O H Emerson and G A Emerson *Ibid* 113 319 (1936)

³⁰ A R Todd, F Berge and T S Work *Biochem J* 31 2057 (1937)

³¹ J C Drummond, E Slinger and R J MacWalter *Ibid* 29 408 2510 (1935)

³² J C Drummond and A A Hoover *Ibid* 31 1802 (1937)

³³ A R Moss and J C Drummond *Ibid* 32 1903 (1938)

³⁴ H M Evans, O H Emerson and G A Emerson *J Biol Chem* 113 319 (1936)

isomer are recovered. The γ isomer has been isolated from a different source by the same technique.³⁵

An efficient separation of the α and β tocopherols is also achieved by the adsorption method.

5 Properties

The α , β and γ isomers of tocopherol are oils which have not been obtained in the crystalline state. Certain esters, however, such as the

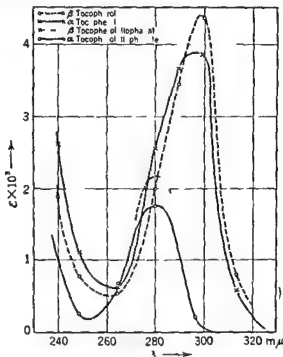


Fig. 23—Absorption spectra of α and β tocopherol and of their allophanates (H. Rudy in W. Stepp, *Ernährungslehre*.)

allophanates *p*-nitrophenyl urethanes and 3,5-dinitro benzoates are crystalline. The tocopherols have a characteristic absorption spectrum in the ultraviolet with a maximum at 295 $m\mu$ which is displaced to 285 $m\mu$ in the esters, such as the acetates. The extinction coefficient for α tocopherol, for example, is $E_{1\text{ cm}}^{1\%} =$ approximately 77 while the coefficient of the esters is reduced to approximately 42 (Fig. 23).

In the absence of oxygen the vitamins E are stable to heat treatment up to 200° C. and are not affected by sulfuric or hydrochloric acid up to 100° C.

³⁵ O. H. Emerson, G. A. Emerson and H. M. Evans, *Science* 83: 41 (1936).

Alkali destroys the vitamins of this group only very slowly, so that they can be obtained by alkaline saponification. The tocopherols are, however, quite sensitive to oxidation, which process destroys the biological activity.

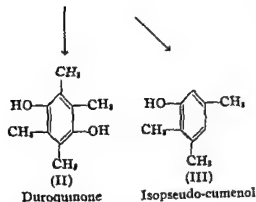
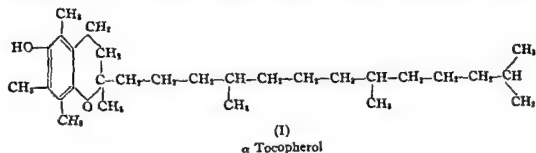
The vitamins E are soluble in all lipid solvents, but are insoluble in water. They are fairly stable to visible light, but are destroyed readily by ultraviolet light.³⁶

The tocopherols are effective antioxidants, the γ isomer being more effective than the β isomer, which in turn is more effective than the α isomer.³⁷ Thus the antioxidant power is the reverse of the vitamin activity and is dependent upon the presence of a free phenolic hydroxyl group, which is not necessarily important for the vitamin action (see page 449).

6 Chemical Constitution

(a) α -Tocopherol

α Tocopherol has the empirical formula $C_{29}H_{50}O_2$. One of the oxygens is present in the form of a free hydroxyl group since the vitamin readily forms esters^{38, 39, 40} and ethers. The phenolic character of this hydroxyl group



³⁶ J. C. Drummond, E. Singer and R. J. MacWalter *Biochem. J.* 29, 456, 2510 (1935)

³⁷ H. S. Olcott and O. H. Emerson *J. Am. Chem. Soc.* 59, 1008 (1937)

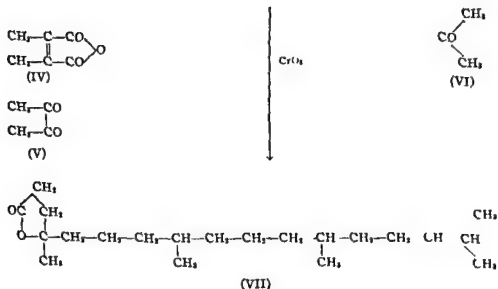
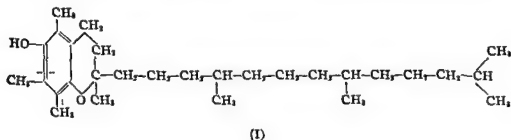
³⁸ H. S. Olcott *J. Biol. Chem.* 107, 471 (1934)

³⁹ H. S. Olcott *Ibid.* 110, 695 (1935)

⁴⁰ H. S. Olcott and H. A. Matill *Ibid.* 104, 423 (1934)

was suspected on the basis of the change of the absorption spectrum upon esterification⁴¹ Upon pyrolysis α tocopherol yields⁴² duroquinone (II), while isopseudo cumenol (pseudo cumenol 6) (III) is obtained by heating with hydriodic acid⁴³ Upon hydrogenation four mols of hydrogen are absorbed⁴³

Further insight into the structure of α tocopherol was obtained by oxidative degradation with chromic acid whereby the following reaction products were isolated dimethyl maleic anhydride (IV) diacetyl (V), acetone (VI), a C_{21} lactone (VII), a C_{18} ketone (VIII) and a C_{18} acid (IX)⁴⁴

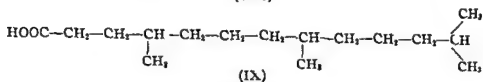
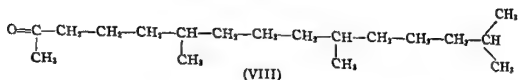


⁴¹ W. John, *Z. physiol. Chem.* 250 11 (1937); W. John, E. Dietzel and P. Günther *ibid.* 252 708 (1938); *Naturwissenschaften* 26 366 (1938); W. John, *Z. physiol. Chem.* 252 2 2 (1938).

⁴² E. Fernholz, *J. Am. Chem. Soc.* 59 1154 (1937).

⁴³ F. Bergel, A. R. Todd and T. S. Work, *J. Chem. Soc.* 1938 753.

⁴⁴ W. John, *Z. physiol. Chem.* 250 11 (1937); W. John, F. Dietzel and P. Günther *ibid.* 252 208 (1938); *Naturwissenschaften* 26 366 (1938); W. John, *Z. physiol. Chem.* 252 272 (1938).



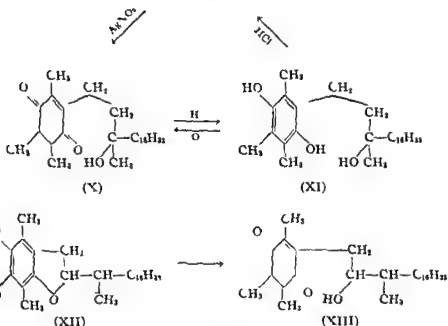
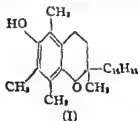
Of these, the C_{21} lactone is of greatest importance. The hydroxy acid from which the lactone is derived, lactonizes readily thus indicating that the hydroxyl group is in either γ - or δ position to the carboxyl group. The methyl ester of the hydroxy acid could not be oxidized to a keto acid which proves that the hydroxyl group is tertiary. Additional evidence for this fact is furnished by the difficulty encountered upon attempted esterification of this hydroxyl group. The C_{16} acid (IX) served for a determination of the side chain methyl groups, which indicated the presence of three. In analogy to many naturally occurring compounds of the terpene family which follow the isoprene rule, formula (IX) was postulated for the C_{16} acid and formula (I) for α tocopherol. The correctness of this formula at least as far as the structure of the aliphatic side chain is concerned, is evident from the successful synthesis of this vitamin from phytol.

The formula of α tocopherol (I) shows the presence of a chroman ring system. That such a ring system and not a coumaran structure occurs was proved⁴⁴ by careful oxidation with ferric chloride or with silver nitrate which yielded a yellow quinone (X).⁴⁵ This undergoes reduction to a hydroquinone, which is a hydroxy quinol ('Tocopheryl quinol') (XI). Upon esterification of the phenolic hydroxyl groups the remaining aliphatic hydroxyl group was investigated and showed all the properties of a tertiary hydroxyl group in oxidation and esterification experiments. A coumaran compound (XII) on the other hand, should have given a secondary hydroxyl group (XIII). The hydroxy quinol (XI) can be reconverted into α tocopherol by treatment with strong mineral acids.

The tocopherols have three asymmetric centers namely at carbon atoms 2, 4' and 8'. It is still an open question whether or not each of the naturally occurring tocopherols represents one of the eight possible isomers. Available evidence indicates that the natural products may be racemic about all three asymmetric centers.⁴⁵

⁴⁴ P. Karrer, R. Lischer, H. Fritzsche, H. Keller, B. H. Ringier and H. Salomon, *Helv. Chim. Acta* 21, 131 (1938).

⁴⁵ P. Karrer, H. Koenig, B. H. Ringier and H. Salomon, *Ibid.* 22, 1139 (1939).



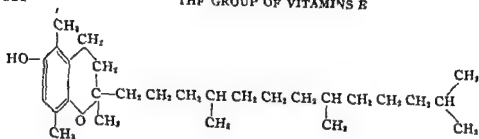
(b) *β* Tocopherol

β Tocopherol has the empirical formula $C_{25}H_{45}O_2$ and thus differs from the empirical formula of *α* tocopherol only by one CH group. Upon pyrolysis of the *β* compound, trimethyl hydroquinone^{47 48} (*ψ* cumoquinol) (II) resulted and upon cleavage with hydriodic acid *p* xyleneol (III) was obtained. Oxidation of *β* tocopherol with chromic acid yielded the same C_{21} lactone (IV)⁴⁹ which was obtained from the *α* isomer. *β* Tocopherol is thus the lower homolog of *α* tocopherol with only two methyl groups in the aromatic ring of the molecule. These two methyl groups are in *p* position to each other. *β* Tocopherol has therefore the structure (I) which was proved by synthesis.

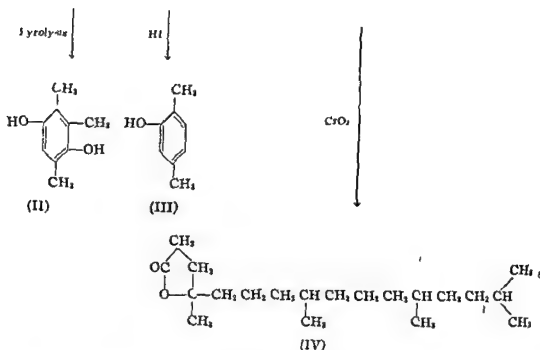
⁴⁷ W. John, *Z. physiol. Chem.* 250 1 (1937). W. John, I. Dietzel and P. Guntber, *Ibid.* 252 104 (1938). *Nat. wiss. schafften* 26 386 (1938). W. John, *Z. physiol. Chem.* 252 929 (1938).

⁴⁸ F. Bergel, A. R. Todd and T. N. Work, *J. Soc. Chem. Ind.* 56 104 (1937).

⁴⁹ O. H. Emerson, *J. Am. Chem. Soc.* 60 141 (1938).



(I)



One position in the benzene nucleus of β tocopherol is not substituted. This fact could be proved by the introduction of an allyl group by means of allyl bromide and zinc chloride⁵⁰

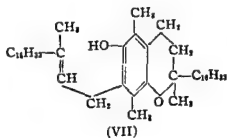
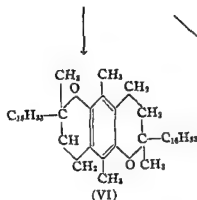
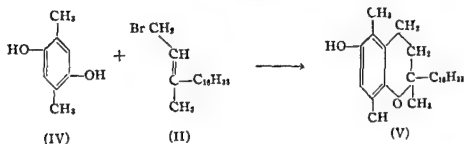
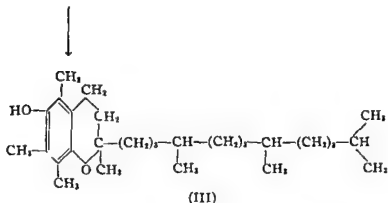
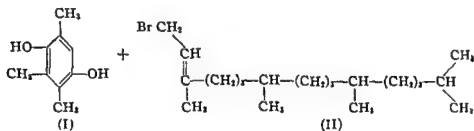
(c) γ Tocopherol

γ Tocopherol has the empirical formula $C_{55}H_{105}O_2$ and is therefore an isomer of β tocopherol. Pyrolysis yields the same compound as does the pyrolysis of β tocopherol, namely, trimethylhydroquinone. Oxidation with chromic acid yields dimethylmaleic anhydride⁵¹. γ Tocopherol is thus *o* xylo tocopherol (I).

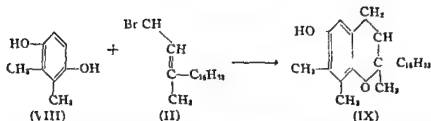
⁵⁰ P. Karrer, R. Escher, H. Brittsche, H. Keller, B. H. Ringier and H. Salomon, *Helv. Chim. Acta* 21, 939 (1938).

⁵¹ O. H. Emerson and L. I. Smith, *J. Am. Chem. Soc.* 62, 1869 (1940).

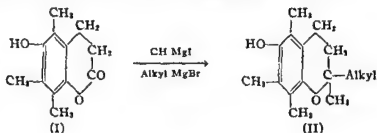
zoates, instead of the free quinols are used for the condensation¹⁹ Subsequently the ester group is removed by hydrolysis



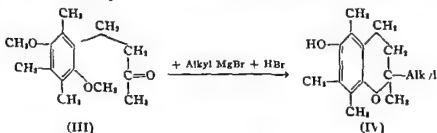
¹⁹ A. Jacob, M. Steiger and A. R. Todd *J. Soc. Chem. Ind.* **57**, 1188 (1938)



An entirely different type of synthesis should be mentioned which has no utility for the preparation of the tocopherols proper, but which has been used successfully for the preparation of similar compounds with different aliphatic side chains. 5,7,8-Trimethyl-6-hydroxy-3,4-dihydrocoumarin is condensed with a mixture of methyl-magnesium halide and alkyl magnesium halide to yield the desired tocopherol homolog⁶⁰



Again a different type of synthesis, not well suited for the tocopherols themselves but attractive for certain of their homologs (IV) is the Grignard reaction of the ketone (III)⁶¹⁻⁶² with an alkyl magnesium halide followed by treatment with hydrobromic acid



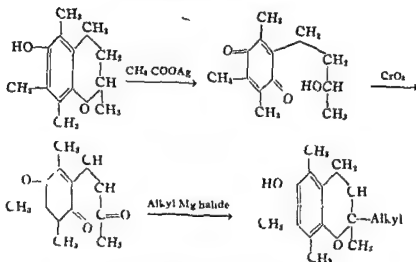
A last method should be mentioned which has been used for the preparation of tocol homologs. This consists of the introduction of a second alkyl group in the 2 position of a 2-methyl-6-hydroxychroman according to the reaction scheme⁶³

⁶⁰ W. John, P. Günther and M. Schmel, *Ber.* 71, 263 (1939).

⁶¹ W. John and P. Günther, *Ibid.* 72, 1649 (1939).

⁶² L. I. Smith, H. E. Ungnade, J. W. Ope, W. W. Prichard, R. B. Carlson and E. W. Kaiser, *J. Org. Chem.* 4, 323 (1939).

⁶³ W. John and M. Schmel, *Ber.* 72, 1653 (1939).



8 Industrial Methods of Preparation

Vitamin E is marketed in the form of concentrates from natural sources and in the form of the racemic *dl* α tocopherol obtained by synthesis. The latter is preferred by the medical profession. The methods used for these processes are those which have already been described in the sections on the isolation and synthesis. The synthetic tocopherol is sold in the form of the acetate which is at least as active as the free phenol and has the advantage of greater stability toward oxidation.⁶⁴ The most important industrial method for the concentration of vitamins E from natural oils is the short path, high vacuum molecular distillation. The natural vitamin F can be stabilized by the addition of antioxidants, such as hydroquinone or ascorbic acid.⁶⁴

9 Biogenesis

Nothing definite is known about the biogenesis of the vitamins E. They are not synthesized in the animal organism, but in the plant. It can be assumed that the synthesis in the plant cells is similar in principle to the laboratory methods for the synthesis of the tocopherols. Phytol occurs in relatively large quantities in the plant, mainly in esterified form in chlorophyll but also in the free state as for example, in germ oils.⁶⁵ There is some evidence for the assumption that vitamins E are synthesized preferentially in the green parts. If this is true, the vitamin is transported into the seeds which usually contain higher amounts than, for example the leaves. Of special interest is the observation that plant embryos for example, wheat

⁶⁴ O. Isler *Helv. Chim. Acta* **21** 1756 (1938)

⁶⁵ A. R. Todd, F. Bergel and T. S. Work *Biochem. J.* **31** 2257 (1937)

germs contain more vitamin E than the seed contained and more per unit weight than the plant will contain at any other stage of its life

The animal organism (for example rat) cannot synthesize vitamins E as has been stated before. Such a synthesis *in vivo* does not occur, even when the starting materials of the laboratory synthesis namely phytol and trimethyl hydroquinone are fed to the animals ⁶⁶

10 Specificity

Synthetic *dl* α tocopherol has the same biological efficacy as the naturally occurring α tocopherol (2-3 mg correspond to one Rat Unit). The β and the γ isomers are only half as active as the α isomer (activity 5 mg) whereas the *m* xylo tocopherol, which has not been found in nature, appears to be almost as active as the α tocopherol (activity 3 mg). Most esters of the tocopherols show excellent vitamin E efficacy, ^{67 68} with the exception of the allophanates which are completely inactive. The acetate propionate and butyrate are said to be even more active than the free vitamin ⁶⁸. The phosphoric acid ester of *dl* α tocopherol upon parenteral administration is more active than the vitamin itself ⁶⁹. Etherification brings about a complete loss of activity.

The tocopherol-quinones are not biologically active ^{70 71 72 73}. The difference in activity (in rats) of the naturally occurring vitamins E points toward a relatively great specificity of this vitamin. This has been substantiated by the preparation and biological evaluation of a great number of compounds of more or less similar structure to the tocopherols. Varying the substituents of the benzene ring it was found that 5,7 dimethyl 8-ethyl tocol ^{74 75} and a diethyl methyl tocol ⁷⁶ are active in a minimum dose of 10 mg while mono methyl tocol and 5,7 diethyl tocol are not active even in doses of 40-50 mg ^{77 78}. Tocol itself ⁷⁹ and 6 desoxy tocol ⁷⁸ at least up to 100 mg doses are also inactive.

⁶⁶ H. M. Evans, O. H. Emerson, G. A. Emerson, L. I. Smith, H. F. Ungnade, W. W. Prichard, P. L. Austin, H. H. Hoehn, J. W. Opie and S. Wawzonek, *J. Org. Chem.* 4, 376 (1939).

⁶⁷ Q. Isler, *Helv. Chim. Acta* 21, 1756 (1938).

⁶⁸ V. Demole, O. Isler, B. H. Ringier, H. Salomon and P. Karrer, *Ibid.* 22, 65 (1939).

⁶⁹ P. Karrer and G. Buemann, *Ibid.* 23, 1137 (1940).

⁷⁰ P. Karrer and A. Geiger, *Ibid.* 23, 455 (1940).

⁷¹ W. John, E. Dietzel and W. Ernte, *Z. physiol. Chem.* 257, 180 (1939).

⁷² P. Karrer, H. Salomon and H. Fritzsche, *Helv. Chim. Acta* 21, 304 (1938).

⁷³ M. D. Wright and J. C. Drummond, *Biochem. J.* 34, 32 (1940).

⁷⁴ P. Karrer, H. Koenig, B. H. Ringier and H. Salomon, *Helv. Chim. Acta* 22, 1139 (1939).

⁷⁵ P. Karrer and O. Hoffmann, *Ibid.* 22, 854 (1939).

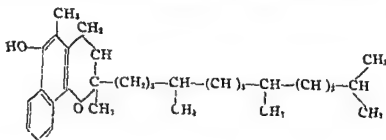
⁷⁶ P. Karrer and O. Hoffmann, *Ibid.* 23, 1128 (1940).

⁷⁷ P. Karrer and H. Fritzsche, *Ibid.* 21, 1234 (1938).

⁷⁸ A. Jacob, F. K. Sutcliffe and A. R. Todd, *J. Chem. Soc.* 1940, 327.

⁷⁹ P. v. Werder, T. Moll and F. Jung, *Z. physiol. Chem.* 257, 129 (1939).

Of special interest is the naphtho tocopherol (I) which shows vitamin E activity in 25 mg doses, but also shows vitamin K activity in doses between 300 and 600 γ ⁸⁰



(I)

The introduction of a double bond into the chroman ring with the formation of a *dl* 3,4 dehydro α tocopherol has no significant influence on the activity, which is about 6 mg⁸¹

The structure of the long aliphatic side chain on the 2 position is of great importance for the biological efficacy. Shortening this chain by one isoprene unit⁸² that is by five carbon atoms or by two such units⁸³ causes loss of the activity (tested up to 40 mg). The same is true for similar substances which contain double bonds in the side chain, for example, for the compound prepared from trimethyl hydroquinone and farnesyl bromide⁸⁴. Compounds which have instead of the long aliphatic side chain only a methyl group or no side chain at all are also inactive⁸⁵. Activity in a 50-60 mg dose has however been claimed for a 2 dodecyl 2,5,7,8 tetramethyl 6 oxy chroman⁸⁶.

A number of other compounds which are less closely related chemically to the tocopherols have been investigated for vitamin E activity. Surprisingly enough some have been found active at least in the rat test which is commonly employed for the determination of vitamins E. All these other compounds, however, are active only in doses of an entirely different order of magnitude. Thus several hydroquinones, their esters and ethers have been found active. *o* and *p* Xylo hydroquinone but not the *m* derivative are active in 100 mg doses⁸⁷. Trimethyl hydroquinone and durohydroquinone, but not trimethyl ethyl hydroquinone, are active at the same level. A naphthoquinone, namely, the 2,3 dimethyl 5,6,7,8

⁸⁰ M. Tishler, I. F. Fieffer and N. L. Wendler, *J. Am. Chem. Soc.* **62**, 1982 (1940).

⁸¹ P. Karrer, R. G. Iegler and G. Schwab, *Helv. Chim. Acta* **23**, 1132 (1940).

⁸² P. Karrer and K. A. Jensen, *Ibid.* **21**, 1600 (1938).

⁸³ P. Karrer and K. S. Yap, *Ibid.* **23**, 581 (1940).

⁸⁴ P. Karrer and K. A. Jensen, *Ibid.* **21**, 1622 (1938).

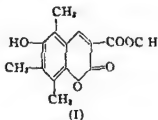
⁸⁵ P. Karrer, R. Escher, H. Fritzsche, H. Keller, B. H. Ringier and H. Salomon, *Ibid.* **21**, 939 (1938).

⁸⁶ W. John, P. Günther and M. Schmeil, *Ber.* **71**, 2637 (1938).

⁸⁷ P. v. Werder, T. Moll and P. Junz, *Z. physiol. Chem.* **257**, 129 (1939).

tetrahydro 1,4 naphthoquinone is reported to be active at high levels⁸⁵ A considerable number of esters and of ethers of trimethyl and of tetramethyl hydroquinone have been tested and practically all of them show some activity at high levels^{87 88 89 90} The simple phenols and phenol ethers are inactive, but *o* allyl phenol *di o* hexenyl phenol and *p* amino *o* allyl phenol are active⁹¹

Chroman itself is active as are the 2,2 diethyl and 2,2 di *n* butyl derivatives but not the corresponding derivatives with odd numbers of carbon atoms 2 5,7,8 Tetramethyl chroman and 2 2,3,7,8 pentamethyl 6 hydroxy chroman are also active Quite a number of coumarins have been tested and were found to be inactive with the exception of compound (I) which is said to be active in doses as low as 20 mg 2 Methyl coumaran, 2 2 7 trimethyl coumaran and 2,3,4 6 7 pentamethyl 5 hydroxy coumaran show activity at about 100 mg doses



11 Determination

The only reliable method for evaluating vitamins E is the biological assay based on a comparison with the synthetic *dl* α tocopherol The physical and chemical methods have a number of shortcomings, the most important of which is that it is impossible to differentiate between the α β and γ isomers, which have different biological efficacies Furthermore the necessity of applying isolation procedures in order to separate interfering substances causes considerable loss

(a) Physical Methods

Spectroscopical Determination^{92 93}—The determination of vitamin E in alcohol or cyclohexane solution by its characteristic absorption spectrum

⁹² F v Werder and T Moll *Ibid* 254 39 (1938)

⁹³ H M Evans O H Emerson and G A Emerson *Science* 88 193 (1938)

⁹⁴ E Fernholz and J Finkelstein *J Am Chem Soc* 60 402 (1938)

⁹⁵ H M Evans O H Emerson G A Emerson L I Smith H E Unzueta W W Prichard F I Austin H H Hoehn J W Opie and S Wawzonek *J O & Cke* 4 376 (1933)

⁹⁶ T Moore and K R Rajagopal *Biochem J* 34 335 (1940)

⁹⁷ W F J Culbertson R R Ridgeway and J C Drummond *Ibid* 34 31 (1940)

with a maximum of 294 $m\mu$ can only be recommended for solutions of the pure or almost pure compounds. The naturally occurring substances with vitamin E activity comprise besides α tocopherol, its esters, which have different absorption maxima and less pronounced extinction coefficients, for example, α tocopherol, $E_{1\text{ cm}}^{1\%}$, 284 $m\mu$ = about 77 α tocopherol acetate, $E_{1\text{ cm}}^{1\%}$, 285 $m\mu$ = about 42. Furthermore, even traces of vitamin A interfere due to the strong absorption characteristics [$E_{1\text{ cm}}^{1\%}$, 328 $m\mu$ = 1725]

Natural materials such as plant oils and the fat extract of animal materials contain many other substances which have no vitamin E activity but exhibit absorption in the region of the vitamin E absorption spectrum and interfere therefore with the spectroscopical determination of the vitamin. Furthermore, any extraction of the vitamin from animal tissues causes some loss. In order to obtain any data it is necessary to saponify the material, for example, the fat extract, germ oil, etc. The saponification destroys further amounts of the vitamin,⁹⁴ unless it is carried out under most carefully controlled conditions.⁹⁵

A modification of the spectroscopical determination of the tocopherols consists in the spectroscopical determination of the oxidation product of the vitamin.⁹⁶ The vitamin is best oxidized⁹⁷ with a 5% solution of silver nitrate in 90% methyl alcohol. The quinone formed shows an absorption maximum at 265 $m\mu$ with an extinction coefficient about four times as high as that of the vitamin itself. Spectroscopical assays are run before and after the oxidation of the vitamin to exclude the values obtained from any previously oxidized material.

(b) Chemical Methods

Ferric Chloride-Dipyridyl Method⁹⁸—This method is based on the oxidation of vitamin E in alcoholic solution by ferric chloride.⁹⁹ An addition of α, α' dipyridyl develops a characteristic red color with the resulting ferrous chloride. A blank is run with each determination. Working in subdued light is recommended, since sunlight tends to cause a development of color in the blank solution.

⁹⁴ A. Emmerie and C. Engel *Rec trav chim* 58 895 (1933)

⁹⁵ A. Emmerie *Ibid* 59 246 (1940)

⁹⁶ W. John *Soc Chem Ind Food Group* 1939 '3

⁹⁷ W. I. J. Cuthbertson, R. R. Ridgeway and J. C. Drummond *Biochem J* 34 31 (1940)

⁹⁸ A. Emmerie and C. Engel *Nature* 142 873 (1938) *Rec trav chim* 57 131 (1938)

J. Waddell and H. Steenbock *J Biol Chem* 80 431 (1928)

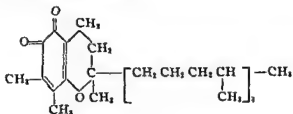
This method is not very specific since the color is given by all reducing compounds. Other substances such as the carotenoids interfere by obscuring the color of the ferrous dipyrldyl. Although it is possible to estimate the vitamin E content of unsaponified oils, these oils often contain other reducing agents which can be removed by saponification. The best results are obtained by hydrolysis with 2 *N* KOH methanol solution for 10 minutes.¹⁰⁰ It has furthermore, been suggested that all interfering substances be adsorbed on, for example floridin which has been activated by treatment with hydrochloric acid.¹⁰¹

A modification of this procedure consists¹⁰² in treating the material to be assayed in a hexane or petroleum ether solution with 85% sulfuric acid followed by centrifuging and washing with dilute alkali. The interfering substances are largely removed and the vitamin E is determined in the remaining solution by ferric chloride and dipyrldyl.

Gold Chloride Method ^{103, 104}—This method consists in the oxidation of vitamin E with gold chloride at about 50° C. The progress of the reaction is followed electrometrically. The equilibrium is but slowly established.

The accuracy of this method like that of the ferric chloride dipyrindyl method is affected by other reducing substances, including carotenoids. For accurate determinations this method requires larger quantities of the vitamin than needed in the ferric chloride dipyrindyl method.

Nitric Acid Method ¹⁰⁵—Vitamin E, upon heating with alcoholic nitric acid yields a characteristic red color. The coloration is caused by the production of the following compound ¹⁰⁶



Since other naturally occurring substances give similar, or at least yellow colors the use of a filter limited to the wave band 450-520 m μ has been

¹⁰ A. Finner, *Rec. trav. chim.* **59**, 246 (1940).

A. Emmerie and C. Engel. *Ibid.* 58: 283 (1939).

¹⁰ W E Parker and W D McFarlane *Can J Resonch* 18 405 (1940)

P Karrer R Fischer H Frutische H Keller B H Ringer and H Salomon *Helv Chem Acta* 21
339 (1938)

¹⁰¹ P. Karrer and H. Keller *Ibid.* 21, 1161 (1938).

¹⁰⁴ M. Furter and R. B. Meyer *Ibid.* 22, 240 (1939).

1. Smith W B Irvin and H F Ungnade *J Am Chem Soc* 61 2424 (1939) *Science* 90 314 (1939)

advocated The color is then measured in one of the common types of colorimeters

A distinct disadvantage of this method is the fact that the simple biologically inactive oxidation product of the tocopherols gives the same coloration with nitric acid Thus, vitamin E destroyed, for example, by air oxidation or by ferric chloride, shows up in this test as being present as vitamin E On the other hand, this method has the advantage that carotenoids do not interfere Appropriately used, this assay procedure is very convenient and rapid ¹⁰⁷

(c) *Biological Methods*

Fertility Test on Rodents ^{108 109} For biological determinations of the vitamin E, female animals (rats) are usually preferred, since they can be essentially healed several times from the avitaminosis, whereas in males (rats) the damage done by the absence of the vitamin is irreparable Rats are used usually in these tests, although occasionally other animals, for example, the rabbit, ¹¹⁰ have been recommended Female virgin rats preferably are used ^{111 112} (at about 30 to 40 days after weaning), since the initial storage of the vitamin in the young can be controlled through placental and mammary transfer

The tests can be carried out by prophylactic or by curative methods, but the latter is considered much more reliable The criterion is the proof of the occurrence of gestation and resorption of the fetuses This can be accomplished by following the weight increase and decrease, respectively, after mating Conception is proved by the plug, that is the bouchon vaginale Successful implantation of the ova can be verified by the placental sign, that is detection of blood in the vagina between the ninth and thirteenth days after mating The percentage fertility is measured in this test An improved method which is based on a graded response of the animals consists in the autopsy of all animals at the tenth day of pregnancy The evaluation comprises a determination of the weight of the uterus, both before and after removal of living fetuses, dead fetuses and resorption sites The results of this latter method are expressed in the so called 'uterine index'

¹⁰⁷ H E Ungnade and L I Smith *J Org Chem* 4 337 (1939)

¹⁰⁸ H M Evans and G O Burr *Proc Natl Acad Sci U S A* 11 334 (1925) H V Diccott and H A Matell *J Biol Chem* 104 423 (1934)

¹⁰⁹ H M Evans and G O Burr *Mem Univ Calif* No 8 (1925)

¹¹⁰ C G Mackenzie and E V McCollum *J Nutrition* 19 345 (1940)

¹¹¹ A L Bacharach E Alchorne and H E Glynn *Biochem J* 31 2287 (1937)

¹¹² A L Bacharach and E Alchorne *Ibid* 32 1298 (1938)

Fertility Test on *Daphnia* The use of *Daphnia magna* the transparent crustacea as a test animal has been suggested for the quantitative estimation of vitamin E¹¹³ The criterion in this assay procedure is the growth of the ovaries and of the parthenogenic embryos in the brood sac

12 Standards

Synthetic racemic alpha tocopherol acetate has been adopted as the international standard for vitamin E and it has been recommended that the International Unit for vitamin E be defined as the specific activity of 1 mg of the standard preparation This quantity is the average amount which when administered orally, prevents resorption gestation in rats deprived of vitamin E¹¹⁴ The international standard is issued in the form of a solution in olive oil of which one International Unit is contained in 0.1 g

One Rat Unit or the fertility dose is the smallest amount of vitamin E which when given *per os* daily to resorption sterile female rats for the entire period of gestation (21 days) brings about in 50% of the animals the birth of at least one living young

One Rat Unit or the fertility dose is equal to 2-3 mg of α tocopherol

The Pacini Linn Unit is equal to $\frac{1}{10}$ of the fertility dose

The Bomskow Rat Unit¹¹⁵ is the amount of vitamin E which when administered once during the first eight days of pregnancy prevents resorption

13 Physiology of Plants and Microorganisms

The present day knowledge of the significance of the occurrence of vitamins E in plants is very meager It must be assumed that the tocopherols play a very definite role in the plant cells and that this role is not primarily concerned with the antioxidative properties of the compounds It is probably quite significant that the seeds usually contain higher amounts than any other part of the living plant On the other hand the germs for example wheat germs contain even higher amounts The possibility of an influence upon cell growth must therefore be considered

Not all organisms contain tocopherols The microorganism *Phycomyces*, for example does not seem to contain any at all as evident from electro metric titration with gold chloride¹¹⁶

¹¹³ A. Viehoever and I. Cohen *Am. J. Pharm.* 110: 29 (1938) A. Viehoever *J. Nat. l. Official Ag. Chem.* 22: 715 (1939)

¹¹⁴ F. M. Hume *Nat. l.* 148: 47 (1941)

¹¹⁵ L. Bomskow *Arch. exp. Path. Pharmacol.* 190: 12: 13 (1939)

¹¹⁶ W. H. Schojfer and S. Blumberg *J. Biol. Chem.* 174: 344 (1948)

Vitamin F when injected into higher plants in olive oil solution exerts an inhibitory effect upon the growth (experiments with *Melandrium album* (Miller) Carke)¹¹⁶

14 Animal Physiology

(a) Metabolism of Vitamins E

Vitamins E and their esters are easily absorbed from the intestinal tract especially in the presence of bile acids¹¹⁷ The vitamin is, however, not well utilized when given parenterally¹¹⁸ The vitamin is taken up by the blood in which its presence can be demonstrated The blood of female rats contains approximately 56 γ and that of male rats 64 γ of tocopherols per 10 cc of serum¹¹⁹ The blood level of rats can be increased up to 100 γ by oral administration of the vitamin Upon an intake of esters for example the acetate, the free vitamin appears in the blood

No significant excretion of vitamin E is observed¹²⁰ as long as normal doses of the vitamin are fed The intake of excess doses causes the excretion of a certain amount in the feces,^{119 121} but only traces are found in the urine¹¹⁹ This means that the vitamin is inactivated in the organism probably by an oxidation mechanism Since, furthermore no simple oxidation products of the tocopherols are excreted, the vitamin molecule must be broken down in the organism (rats)¹¹⁹

Vitamins E are stored to a small but significant extent in animal body fats^{119 122} in the muscles,^{119 1} and especially high amounts are found in the placenta and in the anterior lobe of the pituitary gland Vitamin E is secreted in the milk,^{1 3} and in the eggs of birds

The liver of horse and cattle contains unusually high amounts,^{1 4} but no vitamin E is stored in the liver of rats even on excessive dosage of the vitamin^{119 125}

(b) Physiological Action of Vitamins E

Much work and much speculation concerning the physiological action of the vitamins E arose when the constitution of the vitamins became known

¹¹⁷ J D Greaves and C L A Schmidt *Proc Soc Exptl Biol Med* 37 40 (1937)

¹¹⁸ M Goettsch and A M Pappenheimer *J Nutrition* 22 463 (1941)

¹¹⁹ A Emmerie and C Engel *Rec trav chim* 58 895 (1939) W F J Cuthbertson R R Ridgeway and J C Drummond *Biochem J* 34 34 (1940)

¹²⁰ C S McArthur and E M Watson *Can Chem Proc Ind* 23 310 (1939)

¹²¹ A Juhasz Schaffer *Arch path Anat (Virchow's)* 281 53 (1931)

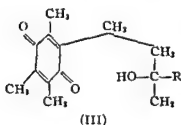
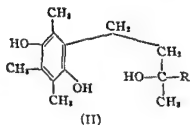
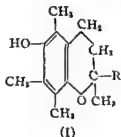
¹²² H M Evans and G O Burr *Mem Univ Calif No 8* (1927)

¹ M M O'Brien *Biochem J* 32 1474 (1938)

¹⁴ P Karrer W Jaeger and H Keller *Helv Chim Acta* 23 464 (1940)

¹²⁵ T Moore A J P Martin and K R Rajagopal *Soc Chem Ind Fed Gr* 1939 11

The fact that they are phenols and that they are antioxidants led to the supposition that vitamins E may take part in some oxidation reduction mechanism. From a chemical standpoint it would be conceivable to assume that the vitamin acts in the following manner



No support for this theory can however be offered. The quinone (III) which can be obtained by oxidation of the vitamin is completely inactive biologically^{126, 127, 128, 129}. Thus a reduction to the quinol (II) in the cells seems quite improbable.

The many symptoms which can be observed during a deficiency of vitamin E points to the assumption that vitamin E is of importance to the tissues as a whole. Unconfirmed experimental findings to the effect that the organism of vitamin E depleted animals contains unsaponifiable material which upon injection into rats causes the symptoms of a vitamin E deficiency, have tentatively been interpreted as indicating a disturbance of some metabolic processes perhaps of the fat metabolism.¹³⁰

A possible relation of vitamin E to the amino acid metabolism is seen in the fact that during avitaminosis the creatine content of striated muscles is

¹²⁶ P. Karrer and A. Geiger *Helv. Chim. Acta* 23 455 (1940)

¹²⁷ W. John, E. D. Tzel and W. Emte *Z. physiol. Chem.* 257 180 (1939)

¹²⁸ P. Karrer, H. Salomon and H. Fritzsche *Helv. Chim. Acta* 21 309 (1938)

¹²⁹ M. D. Wright and J. C. Drummond *Biochem. J.* 34 32 (1940)

¹³⁰ B. A. Kudrjashov *Bull. biol. et med. et ph. URSS* 3 279 (1937)

greatly reduced¹³¹ in rabbits¹³² and in rats¹³³ and that the urinary excretion of creatine is considerably increased¹³¹ ¹³⁴ while the excretion of creatinine remains unchanged. A marked reduction of the creatine content in the urine is observed following the administration of vitamin E. No such changes have, however, been found in the urine of man suffering from progressive muscular dystrophy, amyotonia congenita and similar neuro-muscular disturbances.¹³⁵

The primary physiological action of vitamin E is apparently to direct certain activities of the cell nucleus.^{136, 137} This conception of the mechanism of the vitamin E action, although much disputed, has proved to be of extreme value in an attempt to correlate the various experimental facts and observations of the many different symptoms of vitamin E deficiency.

The role which vitamin E plays in the metabolic activities of the cell nucleus cannot as yet be defined exactly. It seems that the vitamin function is more intimately concerned with the processes of cell maturation and differentiation than with mitosis. Thus the rate of growth of the Walker 256 mammary carcinoma is not essentially affected by continuous maintenance in vitamin E deficient rats.¹³⁸ On the other hand, an increase in growth has been demonstrated in *in vitro* experiments with liver, spleen, heart and periosteal tissue cultures upon addition of vitamin E.¹³⁹ Retarded growth has as in the case of deficiencies of the other vitamins also been observed during vitamin E deficiency.¹⁴⁰ The considerable decrease in the weight of the testis even when calculated in relation to the reduced body weight is, however, specific for this vitamin.¹⁴¹ In accordance with the conception that vitamin E is of special influence to all those tissues in which cellular proliferation and differentiation proceed at a high speed, it has been found that the application of this vitamin is especially useful, for example, to effect growth of prematurely born babies or to heal skin wounds in rats.¹⁴ ¹⁴² Beneficial effects in the latter case have been observed upon oral or local application.

¹³¹ M. Goettsch and E. F. Brown, *J. Biol. Chem.* 97, 549 (1932).

¹³² C. G. Mackenzie and E. V. McCollum, *J. Nutrition* 19, 345 (1940).

¹³³ F. Verzar, *Schweiz. med. Wochenschr.* 69, 738 (1939).

¹³⁴ S. Morgulis and H. C. Spencer, *J. Nutrition* 11, 573 (1936).

¹³⁵ W. Fleischmann, *Proc. Soc. Exptl. Biol. Med.* 46, 94 (1941).

¹³⁶ A. Jukasz-Schäffer, *Arch. path. Anat. (Virchow's)* 281, 33 (1931).

¹³⁷ K. E. Mason, *Am. J. Anat.* 52, 153 (1933).

¹³⁸ K. E. Mason, *Soc. Chem. Ind. Food Group* 1939, 31.

¹³⁹ A. Jukasz-Schäffer, *Arch. path. Anat. (Virchow's)* 281, 35 (1931).

¹⁴⁰ H. Blumberg, *J. Biol. Chem.* 108, 227 (1935).

¹⁴¹ A. M. Copping and V. Korenschevsky, *Soc. Chem. Ind. Food Group* 1939, 44.

¹⁴² G. Léránth and L. Frank, *Orvosi Hetilap* 80, 778 (1936). *Chem. Abstracts* 30, 7634 (1936).

¹⁴³ F. R. Adamstone, *J. Morphol.* 52, 47 (1931).

The histopathological changes which have been observed to occur in the testes of vitamin E depleted rats are largely responsible for the conception of the function of vitamin E. The chromatin of the spermatozoa undergoes a typical change, a lysis and the nuclei become crescentic. Upon further development of the deficiency disease the mature and immature cells the permatogonia, spermatocytes and spermatozoa fuse into giant cells with many nuclei. Besides chromatolysis there appears to be an interference in the formation of chromatin.

Similar effects are observed in the fetus. The earliest signs of a vitamin E deficiency in the embryo is observed in the hematopoietic tissue the growth of which is disturbed. The mesodermal tissues where rapid cellular activity prevails are similarly affected. The symptoms of vitamin E deficiency to be discussed in the next section namely nervous lesions and degenerative lesions in the muscles seem to be related to the cerebral cortex (observations on rats) a tissue of high cellular activity.

All experimental evidences lead to the conclusion that secondary to the lesions in the gonads and in the neuro muscular system and probably as the result of these rather than as their cause abnormalities occur in the pituitary and thyroid glands. In young vitamin E depleted rats a change in the fur has been observed which is considered characteristic for hypophysectomized animals.¹⁴⁴ ¹⁴⁵ In the anterior lobe the acidophil and basophil cells degenerate and show no distinct granulation.¹⁴⁶ The histological appearance of the anterior lobe is quite similar to the degeneration observed in the case of castrated animals. Actually many of the symptoms observed as the result of a vitamin E deficiency resemble very closely the syndromes of a hypophysectomy.¹⁴⁷ ¹⁴⁸ The changes observed in the testes are however dissimilar.¹⁴⁹ Vitamin E deficiency causes a degeneration of the germinative tissue whereas upon dysfunction of the pituitary the interstitial tissue is degenerated. In male rats deprived of vitamin E the pituitaries were found to contain a greater gonadotropic action than found in the glands of rats fed with vitamin E.¹⁵⁰ ¹⁵¹ In female rats deprived of vitamin E the pituitary contains a decreased amount of luteinizing hormone.¹⁵² Nevertheless the important symptoms of vitamin E deficiency, namely muscular dystrophy gestation resorption and testicular degenera-

¹ F Verzár Abderhaldt's Handb. biol. Arbeitsmeth. den. Abt. 5 Pt. 36 No. 8 1263 (1937)

¹⁰ F Verzár and E Kokas Arch. ges. Physiol. (Pflügers) 227 511 (1931)

¹¹ M. M. O. Barrie Lancet II 51 (1937)

¹² M. M. O. Barrie J. Soc. Chem. Ind. 53 1033 (1936)

¹³ F Verzár Arch. ges. Physiol. 227 499 (1931)

¹⁴ K. E. Mason Am. J. Anat. 52 153 (1933)

¹⁵ W. O. Nelson Anat. Record 56 241 (1933)

¹⁶ J. C. Drummond Soc. Chem. Ind. Food Group 1939 27

¹⁷ I. W. Rowlands and P. Singer J. Physiol. 86 323 (1936)

ture which has been found in rats,^{170 171 172 173 174 175} rabbits,^{176 177 178} dogs,¹⁷⁹ guinea pigs^{180 181 182 183} and chicks¹⁸⁴ Before the overt symptoms of such a dystrophy appear, the following syndromes can be detected¹⁸⁵ increase in the concentration of water and of chlorides in the muscles, decrease in the maximum strength and focal hyaline necrosis of muscle fibers These lesions are accompanied by degenerative changes in the central nervous system,¹⁸⁶ histologically evident by degeneration of nerve cells in the spinal cord¹⁸⁷ In cases of severe avitaminosis, paralysis first of the hind and then also of the fore limbs is found in young¹⁸⁸ and in old rats Carpopedal spasms and convulsions have been observed in the final stages when the mortality is 100%¹⁸⁹

Another pathological change observed in rats deprived of vitamin E is a considerable enlargement of the thymus both in male and in female animals¹⁹⁰ The changes which occur in the pituitary, the thyroids and the kidney have already been discussed In the young vitamin E deficient rat also an uncalcified skull has been observed

In beef cattle and in milk cows, sterility and early abortion have successfully been treated with vitamin E and the existing evidence leads to the conclusions that some of these animals suffered from a true E avitamins

¹⁷⁰ See however M Goettsch and J Ritzmann *J Nutrition* 17 371 (1939)

¹⁷¹ H M Evans and G O Burr *J Biol Chem* 76 273 (1938) H Blumberg *Ibid* 108 227 (1935)
G O Burr W R Brown and R L Mosely *Proc Exptl Biol Med* 36 780 (1937) A Ringstead
Biochem J 29 788 (1935)

¹⁷² H S Olcott *J Nutrition* 15 221 (1938)

¹⁷³ H M Evans and G O Burr *J Biol Chem* 76 273 (1938)

¹⁷⁴ L Einarson and A Ringstead *Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats* Copenhagen 1938

¹⁷⁵ M D Lipshutz *Rev neurol* 65 221 (1936) J Morelle *Compt rend soc biol* 108 804 (1931)

¹⁷⁶ H S Olcott and H A Matill *J Biol Chem* 104 423 (1934)

¹⁷⁷ C G Mackenzie and E V McCollum *J Nutrition* 19 345 (1940)

¹⁷⁸ S Morgulis *Monographie Actualites Scientifiques et Industrielles* Paris 1938 p 74

¹⁷⁹ S G Morris *Science* 90 424 (1939)

¹⁸⁰ H D Anderson C A Elvehjem and J E Conce *Proc Soc Exptl Biol Med* 42 750 (1939)

¹⁸¹ K M Brinkhous and F D Warner *Am J Path* 17 81 (1941)

¹⁸² M Goettsch and A M Pappenheimer *J Exptl Med* 54 145 (1931)

¹⁸³ H S Olcott *J Nutrition* 15 221 (1938)

¹⁸⁴ N Shimotori G A Emerson and H M Evans *Science* 90 89 (1939) *J Nutrition* 19 547 (1940)

¹⁸⁵ E L Wood and H M Hines *Proc Soc Exptl Biol Med* 36 786 (1937)

¹⁸⁶ H Dam and J Glavind *Naturwissenschaften* 28 207 (1940) *Nature* 143 810 (1939)

¹⁸⁷ G C Knowlton and H M Hines *Proc Soc Exptl Biol Med* 38 655 (1938) 42 133 804 (1939)

¹⁸⁸ D Lipshutz *Rev neurol* 65 221 (1936) L Einarson and A Ringstead *Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats* Copenhagen 1938

¹⁸⁹ M Ekblad and G Wohlfart *Z ges Neurol Psychiat* 168 144 (1940)

¹⁹⁰ H M Evans and G O Burr *J Biol Chem* 76 273 (1938)

¹⁹¹ M M O Barrie *Lancet* II 251 (1937)

¹⁹² A M Copping and V Korenschevsky *Proc Chem Ind Food Group* 1939 44

¹⁹¹ ¹⁹² There is also considerable evidence¹⁹³ that administration of vitamin E is of value in the treatment of the infectious abortion in cows, caused by the *Bacillus abortus* (Bang) probably due to the fact that vitamin E increases the resistance of the animals against infection¹⁹⁴

In sows barrenness has also successfully been overcome by vitamin E therapy¹⁹⁵

The pathological changes observed on rats are essentially similar to the changes observed in birds. Eggs of hens reared on a vitamin E deficient diet cannot be hatched successfully. In the embryos a lethal ring develops in the blastoderm from cell proliferation in the mesoderm. The organism dies from starvation and hemorrhages after the blood vessels of the blastoderm are choked off¹⁹⁶. Hatchability is greatly improved upon adding vitamin E to the chicken feed¹⁹⁷ ¹⁹⁸ ¹⁹⁹. In the male fowl degeneration of the testis has been observed¹⁹⁸ ¹⁹⁷. In chicks a vitamin E deficiency causes alimentary exudative diathesis followed by increased capillary permeability and muscular dystrophy²⁰⁰. Nutritional encephalomalacia of the chick is caused by vitamin E deficiency²⁰¹.

The question as to whether or not an E avitaminosis occurs in man has not definitely been decided. Considerable evidence exists for the fact that vitamin E may exert a beneficial influence in certain cases of habitual abortion²⁰² ²⁰³ and possibly also of threatened abortion and premature separation of the placenta. Certain forms of toxemia in pregnancy²⁰⁴ have also been reported to respond favorably to a treatment with vitamin E. It has furthermore been stated that administration of vitamin E is beneficial

¹⁹¹ F. Vogt Möller and F. Bay *Münch tierärztl Wochschr* 82 637 (1931) *Vet J* 87 165 (1931) 90 288 (1934)

¹⁹² A. Ande sen *Medlemsblad dansk Dy læg foren* 17 113 (1934) H. Lehmknecht *Ernähr Wochschr* 1938 367 J. P. Tutt *Vet J* 89 416 (1933)

¹⁹³ R. Moussu *Compt rend* 201 12 8 (1935)

¹⁹⁴ A. E. Lange *Tier ärztl Rundz* 1938 239

¹⁹⁵ R. W. Lentz *Berlin tier ärztl Wochschr* 1938 201

¹⁹⁶ P. R. Adamstone *J Morphol* 52 47 (1931)

¹⁹⁷ L. P. Card *Poultry Sci* 8 3 8 (1919)

¹⁹⁸ G. I. Barnum *J Nutrit* 9 621 (1935)

¹⁹⁹ F. Ender *Z. Vitam ino sch* 4 106 (1935) see however C. R. Holmes and W. W. Craven *Po il y Sci* 19 303 (1940)

²⁰⁰ H. Dam and J. Glavind *Nat w ssenschaft* 28 207 (1940) *Nat re* 143 810 (1939)

²⁰¹ A. M. P. ppenheimer, M. Goettsch and E. Junghert *Connecticut Agric Ex p Stat Bull* 229 (1939)

²⁰² F. V. g. t Möller *Lancet* 221 187 (1931) *Acta Obstet Gyn Scand* 13 219 (1933) *Klin Wochschr* 15 1883 (1936)

²⁰³ D. W. Curr e *Brit J Med* 11 1218 (1937) J. Gierhake *Arch Gynäkol* 156 348 (1933) A. Juhász-Schäffer *Egeb sw Med* 45 129 (1933) H. Martius *Med Welt* 1937 407 E. V. Shute *J Obstet Gynecol Brit Emp* 42 10 1 1095 (1917) 43 4 (1938) *Am J Obstet Gynecol* 33 427 (1917) F. M. Watson *Canad Med Assoc J* 34 131 (1936) F. M. Watson and W. P. Tew *Am J Obstet Gynecol* 31 257 (1933) J. Young *Brit J Med* 1 1 103 (1917)

²⁰⁴ F. V. Shute *Am J Obstet Gynecol* 33 479 (1937) J. Young *Brit J Med* 1 1 111 (1917)

in cases of muscular dystrophy²⁰⁵,²⁰⁶ amyotrophic lateral sclerosis²⁰⁵,²⁰⁷ or myelopathy from pernicious anemia, neuromuscular syndromes, roaring sensations in the ears, anorexia²⁰⁹ and primary fibrositis¹⁰. The information gained from these reports must, however, be regarded as provisional and all clinical use of vitamin E is still considered purely experimental.

(a) Clinical Test Methods

Determination of Vitamin E in Urine *Urinary Creatine Determination*²¹¹—Vitamin E hypovitaminosis can be detected by urine analysis since the creatine output increases rapidly. In rabbits, for example, the daily creatine excretion rises from a normal level of less than 10 mg to over 20 mg. By the time symptoms of muscular dystrophy occur, 10 to 150 mg of creatine are excreted²¹. This method is only of restricted value since many other factors may influence the creatine output.

Determination of Vitamin E in Blood *The Ferric Chloride Dipyrindyl Method* For a successful determination of vitamins E in blood it is necessary to extract the active material. This can, for example, be accomplished by treating the serum with dilute alkali in the cold and in the presence of formaldehyde and ethyl alcohol. The vitamin is then extracted with ether and the ether solution is washed neutral with alkali and acid solutions. By selective adsorption, for example, on floridin,²¹² the carotenoids are removed. The determination of the vitamin E in the remaining solution is then carried out according to the ferric chloride dipyrindyl method. As little as 5 cc of blood can be used for this method. This method fails to be accurate for amounts of vitamin E less than 5 γ .

16 Hypervitaminosis

Vitamins E are non-toxic substances. *E hypervitaminosis is unknown*. Synthetic dl α -tocopherol causes no toxic symptoms in rats even when given orally in doses as high as 50 g per kilogram of body weight. The administration of several grams of vitamin E daily over a period of one to

²⁰⁵ F. Bicknell, *Lancet* I 10 (1940).

²⁰⁶ S. Stone, *J. Am. Med. Assoc.* 114 2187 (1940).

²⁰⁷ I. S. Wechsler, *Ibid.* 114 948 (1940). T. D. Spies and R. W. Vilter, *Southern Med. J.* 33 663 (1940).

²⁰⁸ L. Einarson and A. Ringstead, *Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats*, Copenhagen 1938.

²⁰⁹ T. D. Spies, D. P. Hightower and L. H. Hubbard, *J. Am. Med. Assoc.* 115 297 (1940).

²¹⁰ C. L. Steinberg, *Am. J. Med. Sci.* 3 347 (1941).

²¹¹ O. Folin, *J. Biol. Chem.* 17 469 (1914).

²¹² C. G. Mackenzie and F. V. McCollum, *J. Nutrition* 19 34 (1940).

²¹³ A. Emmerie and C. Fingel, *Rec. trav. chim.* 58 283 (1939).

two months does not result in any adverse symptoms in rats dogs and cats. The functions of the kidneys, the intestines, the nervous system, the muscles and the sex organs are not affected by such doses.²¹⁴

17 Requirements

It has been proved experimentally that vitamin E is necessary for mice rats rabbits guinea pigs dogs chicks and ducks. The crustacea *Daphnia* also needs vitamin E.²¹⁵ Goats can apparently get along without an external supply of this vitamin.¹⁶ The requirements for man are largely unknown as stressed before.

The vitamin E requirement of the rat is different for the male and the female animal. It seems that the male animal needs only about one seventh to one tenth of the amount required by the female. A similar but perhaps more marked difference is observed in mice.

The average daily need of a female rat is from 2-3 mg. of α tocopherol. The minimum fertility requirements of female rats are 15 mg. per kilogram body weight daily. For rabbits the daily antimuscular dystrophy dose is about 1 mg. of α tocopherol per kilogram body weight.¹⁷ In clinical therapy a dose of 6 mg. of α tocopherol per day has been recommended for women.²¹⁸

²¹⁴ V. Demole, *Z. Vitaminsforsch.* 8, 338 (1939).

²¹⁵ A. Vichoever and I. Cohen, *Am. J. Pharm.* 110, 297 (1938).

²¹⁶ G. K. L. Underbjerg, *Iowa State College J. Sci.* 15, 107 (1940).

²¹⁷ C. G. Mackenzie and E. V. McCollum, *J. Nutrition* 19, 345 (1940).

²¹⁸ D. W. Currie, *Soc. Chem. Ind. Food Group* 1939, 77.

VITAMIN H—
BIOTIN

VITAMIN H—BIOTIN

1 Nomenclature

Names

Bios II (Lucas 1924)
Factor X (Boas 1927¹)
Vitamin H (Gyorgy 1931²)
Coenzyme R (Allison Hoover and Burk 1933³)
Bios II b (Miller 1934)
Anti egg white injury factor (Lease and Parson 1934⁴)
Biotin (Kögl 1935⁵)
Factor W (Elvehjem 1936⁶)
Vitamin B₇ (Lunde and Kringstad 1940⁷)
S or Skin Factor (Marshall 1939⁸)

Empirical formula



Efficacy

One gram of biotin methyl ester = 27 000 000 Rat Units
= 25 000 000 000 Sarcharomycetes Units

2 Chronology

- 1901 WILDIERS recognized that yeast needs a special material bios in addition to the known nutrients for optimal growth. This had already been postulated by LIEBIG in 1869
- 1916 BATEMAN⁹ made the casual observation that egg white exhibits a definite toxicity
- 1923 FULMER discovered the multiple nature of bios
- 1924 LUCAS separated bios into two components bios I and II
- 1927 BOAS¹¹ found that certain foods contain an organic substance which protects against egg white toxicity

¹ M. A. Boas *Biochem J* 21 712 (1927)

² P. György *Z. d. all. Fortbild. ng* 28 377 417 (1931)

³ F. E. Allison, S. R. Hoover and D. Burk *Science* 78 217 (1933)

⁴ J. C. Lease and H. T. Parson *Biochem J* 28 2109 (1934)

⁵ F. Kögl *Ber.* 68 16 (1935)

⁶ C. A. Elvehjem, C. J. Koehn and J. L. Olson *J. Biol. Chem.* 115 707 (1936)

⁷ G. Lunde and H. Kringstad *J. Nutrition* 19 31 (1940)

⁸ W. Marshall *J. Invest. Dermatol.* 2 206 (1933)

⁹ W. Marshall *Med. World* 57 101 (1939)

¹⁰ W. G. Bateman *J. Biol. Chem.* 26 63 (1916)

¹¹ M. A. Boas *Biochem J* 21 71 (1927)

- 1928 EASTCOTT¹² isolated bios I in the pure form and identified it with ϵ inositol
 1931 GYÖRGY¹ recognized the necessity of an anti egg white injury factor (vitamin H) for man
 1933 MILLER showed that bios II contained at least two different factors
 1935 KÖGL¹³ isolated biotin one of the compounds of bios II in the pure form
 1940 GYÖRGY MELVILLE BURK and DU VIGNEAUD¹⁴ identified vitamin H with biotin
 1942 DU VIGNEAUD HOFMANN and MELVILLE suggested structural formulas for biotin on the basis of degradation reactions¹⁴

3 Occurrence

Vitamin H occurs in small amounts in all higher animals (mammals and birds) The only tissue found so far which is apparently devoid of this vitamin is the lens of the eye¹⁵ The highest concentrations have been observed in liver¹⁻¹⁶ kidney¹ and eggs¹⁵ while milk¹⁸ contains this vitamin in somewhat smaller concentrations

Vitamin H is found widely distributed in the plant kingdom Vegetables grains, nuts, etc contain considerable quantities¹⁶ Most strains of yeast contain some vitamin H¹⁶ In plants the tips of the roots and of the coleoptiles usually have a somewhat higher concentration than the rest of the plant¹⁷ Relatively high concentrations have been observed in seeds and in pollen¹⁷

Vitamin H occurs predominantly in the free state in fruits and in grasses In grains, nuts and vegetables this vitamin is present partly in a bound form and partly free¹⁹ In yeast and in animal tissues for example, in liver, vitamin H occurs mainly as a chemically bound compound

4 Isolation

As pointed out previously vitamin H occurs in many sources chemically bound and cannot be dissolved by any solvent In order to liberate the vitamin, for example, from yeast,¹⁹ the material is autolyzed from four to six days Liver, on the other hand does not possess the necessary enzyme system for the autolytic liberation of this vitamin²⁰ Vitamin H can now

¹² V Eastcott *J Phys Chem* 32 1094 (1928)

¹³ Kogl *Ber* 68 11 (1935)

¹⁴ I György D B Melville D Burk and V du Vigneaud *Science* 91 743 (1940)

¹⁵ V du Vigneaud K Hofmann and D B Melville *J Am Chem Soc* 64 188 (1942)

¹⁶ Kogl and W an Haselt *Z physiol Chem* 243 183 (1936)

¹⁷ J Junde and H Kringstad *J Nutrition* 19 371 (1940)

¹⁸ I Kogl and A J Haagen Smith *Z physiol Chem* 243 203 (1936)

¹⁹ J O Lampen A A Kline and W H Peterson *Proc Am Soc Biol Chem* 1941 LXIV 10
 Lampen G I Bahler and W H Peterson *J Nutrition* 23 11 (1942)

²⁰ P György *J Biol Chem* 131 733 (1939)

²¹ György R Kuhn and I Federer *Ibid* 131 740 (1939)

ever be liberated by proteolytic digestion of liver^{20 21 22 23} or by hydrolysis with acids,²¹ for example, with 2*N* mineral acids with or without pressure²⁰ In contrast to fresh liver, the vitamin can be liberated from liver powder by hydrolysis at high pressure without addition of acids After the hydrolysis has been achieved according to any of these procedures the solution is filtered and impurities may be removed from the filtrate by precipitation with alcohols or lead acetate The vitamin can be dissolved in acetone from a concentrated filtrate whereby considerable amounts of impurities separate Further concentration has been achieved by precipitation with phosphotungstic acid which precipitates only the impure compound but not the pure vitamin^{24 25} Mercuric chloride⁶ gold chloride and H_2PtCl_4 also precipitate the vitamin from impure solutions The vitamin is however not precipitated by flavianic acid, rufianic acid Reinecke salts picric acid picrolonic acid barium salts⁵ quinine or alkaloids²⁷ Alcohol insoluble salts are obtained from barium or calcium hydroxide

Vitamin H is adsorbed on charcoal (Norit and Carboraffin) but not on fuller's earth aluminum oxide acid clay or benzoic acid Vitamin H can be eluted with a mixture of pyridine methanol and water The methyl ester on the other hand can be purified by adsorption on aluminum oxide^{27a}

Further purification of the vitamin can be accomplished by electro dialysis²⁸ and by high vacuum distillation²⁹

By a combination of these procedures 11 mg crystallized biotin have been obtained from 250 kg of dried Chinese egg yolk²⁹

5 Properties

The free vitamin H is water and alcohol soluble but is relatively insoluble in chloroform ether and petroleum ether The vitamin is essentially heat stable, readily dialyzable and resistant to treatment with acid or alkali It is easily adsorbed on charcoal The isoelectric point appears to be between pH 3 and 3.5 The pure vitamin melts at 230–232° C and the methyl ester at 166–167° C Biotin is optically active $[\alpha]_D^{25} = +92^\circ$

¹ J. G. Lease *Z. Vitaminforsch.* 5: 110 (1936)

² J. G. Lease and H. T. Parsons *Proc. Am. Soc. Biol. Chem. J. Biol. Chem.* 105: 1 (1934)

³ P. György *Ibid.* 119: XLIII (1937)

⁴ J. G. Lease *Z. Vitaminforsch.* 5: 110 (1936)

⁵ P. György, R. Kuhn and E. Lederer *J. Biol. Chem.* 131: 745 (1939)

⁶ C. Drumel and L. Hubert *Arch. sci. (Physiol.)* 46: 141 (1938)

⁷ T. W. Breb and P. György *J. Biol. Chem.* 131: 761 (1939)

⁸ V. du Vigneaud, K. Hofmann, D. B. Melville and P. György *J. Biol. Chem.* 140: 643 (1941)

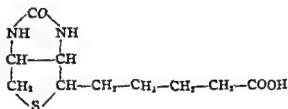
⁹ P. György, D. B. Melville, D. Burk and V. du Vigneaud *Science* 91: 743 (1940)

¹⁰ F. Kögl and B. Tönnes *Z. physiol. Chem.* 242: 43 (1936)

in 0.1 *N* NaOH. It shows no characteristic absorption in the ultraviolet region.³⁰

6 Chemistry

The empirical formula of biotin is $C_{10}H_{16}O_3N_2S$. By means of electrolysis it was shown that vitamin H is an ampholyte with acidic properties.³¹ The presence of a carboxyl group is indicated by the formation of esters such as the methyl ester. Biotin contains a basic group since at pH = 1 the vitamin cannot be extracted with organic solvents.³² The nitrogens are present in urea configuration. By treatment of biotin with barium hydroxide at 140° C. a diamino carboxylic acid is obtained with the loss of one carbon atom and one oxygen atom. By treatment of the diamino carboxylic acid with phosgene the urea group and thereby biotin is resynthesized. The sulfur is present as a thio ether, since a sulfone results from peroxide oxidation. Since biotin does not contain any double bonds it must contain a bicyclic ring system.^{32a} Upon oxidation of the diamino carboxylic acid with nitric acid or alkaline permanganate, adipic acid is isolated.^{33b} One of the carboxyl groups of the adipic acid is the carboxyl group originally present in biotin, since upon Curtius degradation of the biotin methyl ester followed by oxidation of the obtained amine no adipic acid could be isolated.^{32a} On the basis of these and additional evidences,^{32d} biotin has been assigned the structure of 2 keto 3,4 imidazolido 2 tetrahydro thiophene *n* valeric acid (I). This conclusion has been confirmed by a total synthesis of biotin.³⁴



³⁰ V. du Vigneaud, K. Hofmann, D. B. Melville and J. R. Rachele, *J. Biol. Chem.* **140**, 763 (1941).

³¹ T. W. Brich and P. György, *J. Biol. Chem.* **131**, 461 (1939).

³² H. Kringstad and G. Lunde, *Ibid.* **261**, 110 (1939).

³³ F. Kögl and L. Pons, *Z. physiol. Chem.* **269**, 61 (1941); F. Kögl and T. J. de Man, *Ibid.* **269**, 81 (1941); K. Hofmann, D. B. Melville and V. du Vigneaud, *J. Biol. Chem.* **141**, 207 (1941); D. B. Melville, K. Hofmann and V. du Vigneaud, *Science* **94**, 308 (1941).

^{33b} K. Hofmann, D. B. Melville and V. du Vigneaud, *J. Am. Chem. Soc.* **63**, 337 (1941).

³² V. du Vigneaud, K. Hofmann and D. B. Melville, *J. Am. Chem. Soc.* **64**, 188 (1942).

^{32d} Refer to review papers by V. du Vigneaud, *Science* **96**, 455 (1942) and K. Hofmann, *Advances in Enzymology*, Vol. III, Interscience, New York, 1943, p. 280.

³⁴ S. A. Harri, D. E. Wolf, R. Moxingo and K. Folker, *Science* **97**, 447 (1943).

7 Industrial Methods of Preparation

Pure vitamin H is marketed in small quantities prepared by tedious isolation procedures. Concentrates are also manufactured from liver, yeast and certain bacteria. The preparation of the concentrates from liver is coordinated with the manufacture of the factor which prevents the development of pernicious anemia since the residues from this manufacture contain relatively high concentrations of vitamin H.^{22, 21, 16}

8 Specificity

Very little is known about the specificity of vitamin H. The methyl ester is fully active. Benzoylation and acetylation do not effect the activity probably because of the ability of the organism to hydrolyze these derivatives. Vitamin H in its naturally occurring chemically bound form is also essentially active. Inactive however is the biotin symplex with avidin (see page 476).

9 Determination

(a) Biological Methods

There are only biological methods available for the determination of biotin. The most valuable and accurate methods are those in which micro organisms are used and in which the biotin concentration is measured by the growth of yeast or of bacteria or by the amount of acid produced by specific organisms. The historically important bio assay with rats is based upon a secondary deficiency disease brought about by egg white injury and is quite time consuming. The chick can also be used for quantitative assays in a procedure which uses a primary biotin deficiency and which is more expedient than rat assays but less so than the micro biological methods.

Rat Tests *Male albino rats are fed rations which are well balanced except for a large proportion of egg white as the sole source of protein.*^{24, 27} The average time necessary for the appearance of the first skin symptoms is five to seven weeks. A rat growth method has also been used.²³

²² J. G. Leese and H. T. Parsons *Biochem J.* 28: 109 (1934).

²¹ P. György, *J. Biol. Chem.* 131: 733 (1939).

¹⁶ J. G. Leese *Z. V. lammforsch.* 5: 110 (1936).

²⁴ M. A. Bous *Biochem J.* 21: 712 (1927).

²⁷ P. György *J. Biol. Chem.* 131: 733 (1939).

²³ G. Lundén and H. Kringstad *J. Nutrit.* 19: 11 (1940).

Chick Test The chick has been suggested as test animal for the determination of vitamin H since chicks can be depleted readily from this vitamin on a diet low in this constituent³⁰

Coenzyme R Assay⁴⁰ In this test, the increase in respiration of rhizobium is measured upon addition of vitamin H to the culture medium. This method is far more sensitive than the rat assay method for vitamin H.

Yeast Growth Method The growth of special yeast strains such as *Saccharomyces cerevisiae* is observed for example, by measuring the resulting turbidity in a photoelectric colorimeter⁴¹ or nephelometer.⁴

Staphylococcus Growth Test⁴² The response of the addition of vitamin H preparations to a culture medium of *Staphylococcus aureus* has been suggested as a quantitative method for the assay of vitamin H.

Colostridium Butylicum Growth Test⁴⁴ The organism *Colostridium butylicum* has been suggested for vitamin H assays. The growth is measured by turbidity studies. In this test only the free vitamin is measured and not the bound vitamin.

Acidimetric Lactobacillus Test⁴ The amount of lactic acid produced by *Lactobacillus arabinosus* cultivated in a medium containing all the other necessary growth factors can be used as a measure of the vitamin H content.

10 Standards

One "Rat Unit" is the daily dose of a given preparation or foodstuff which in four weeks brings about complete cure of the egg white injury in rats (on a special diet).⁴⁸ This corresponds to an activity of 27 million Rat Units of vitamin H per gram of the methyl ester of biotin.⁴⁷

One *Saccharomyces* Unit⁴³ (S U) is the amount of biotin which produces an increase of 100% in cell growth of a special strain of yeast under defined conditions. One gram of biotin contains 25,000,000,000 *Saccharomyces* Units.

³⁰ D M Hegsted, J J Oleson, R R Mills, C A Elvehjem and E B Hart, *J Nutrition* 20: 599 (1940).
⁴⁰ Ansbacher and M Landy, *Proc Soc Exptl Biol Med* 48: 3 (1941).

⁴¹ F E Allison, S R Hoover and D Burk, *Science* 78: 217 (1933).
⁴² F E Allison and F W Minor, *Soil Sci* 46: 473 (1938).

⁴³ C E Snell, R C Bakin and R J Williams, *J Am Chem Soc* 62: 175 (1940).

⁴⁴ F Kögl and B Tönies, *Z physiol Chem* 242: 43 (1936).

⁴⁵ J R Porter and M J Pelczar, *Science* 91: 276 (1940).

⁴⁶ J O Lampen, A A Kline and W H Peterson, *Proc Am Soc Biol Chem* 1941 LXIV.

⁴⁷ F F Snell and L D Wright, *Ibid* 1941 CLIX.

⁴⁸ I György, *J Biol Chem* 131: 733 (1939).

⁴⁹ P C Rose, K Hofmann, D B Melville and V d Vigne, *Nutrition Abstracts* 92: 1074 (1940).

⁵⁰ F Kögl and B Tönies, *Z physiol Chem* 242: 43 (1936).

11 Physiology of Plants and Microorganisms

Vitamin H is necessary for the growth of certain bacteria, for example, various strains of *clostridium*,^{49 50} *rhizobium*,^{51 52} *staphylococcus*^{53 54 55} etc., and certain yeast strains.⁵⁶ Other bacterial species have been shown to synthesize biotin in varying amounts.⁵⁷ Vitamin H is also a true growth hormone for higher plants. This fact is indicated by the occurrence of increased amounts in plant seeds and in the tips of roots and coleoptiles and has been proved experimentally with isolated pea roots.⁵⁸ The roots of higher plants excrete biotin into the soil.⁵⁹

The role which biotin plays in plants and in microorganisms may be that of a true growth stimulant but an influence upon other factors is by no means excluded. Thus, it has been observed that in yeast biotin increases fermentation more directly than respiration and respiration again more directly than growth.⁶⁰ It has also been shown for *rhizobium* that the respiration can be markedly increased without concomitant growth by the addition of biotin to the culture medium.⁶¹

12 Animal Physiology

(a) Metabolism of Vitamin H

Vitamin H is easily absorbed from the intestinal tract. The naturally occurring bound form seems to be equally well utilized perhaps due to hydrolysis in the intestine. Some destruction appears to occur during the adsorption of biotin since the requirement by subcutaneous administration is only about one fifth of that required for oral administration.

The animal and human organism is apparently able to store a certain amount of vitamin H especially in the liver and the kidneys. On the other hand, newborn infants have practically no such reserves. Similarly the secretion into milk although not negligible is markedly low. Excretion in the feces has been observed but occurs predominantly through the urine.

⁴⁹ W. H. Peterson, L. E. McDanel and E. McCoy *J. Biol. Chem.* **133** LXXV (1940).

⁵⁰ E. E. Snell and R. J. Williams *J. Am. Chem. Soc.* **61** 3594 (1939).

⁵¹ P. M. West and P. W. Wilson *E. xymologia* **8** 152 (1940).

⁵² R. Nilsson, G. Bjälve and D. Burström *Ann. Landw. Hochschule Schwedens* **7** 301 (1939).

⁵³ *Naturwissenschaften* **26** 661 (1938); **27** 389 (1939).

⁵⁴ J. R. Porter and M. J. Pelczar *Science* **93** 576 (1940).

⁵⁵ F. Kögl and W. J. van Wageningen *Rec. trav. chim.* **57** 747 (1938).

⁵⁶ G. A. Hottel, J. O. Lampen and A. M. Pappenheimer *J. Biol. Chem.* **137** 459 (1941).

⁵⁷ E. E. Snell, R. E. Eakin and R. J. Williams *J. Am. Chem. Soc.* **62** 175 (1940).

⁵⁸ M. Landy and D. M. Decker *Proc. Soc. Exptl. Biol. Med.* **46** 449 (1941).

⁵⁹ F. Kögl and A. J. Haagen-Smith *Z. physiol. Chem.* **243** 209 (1936).

⁶⁰ P. M. West *Nature* **144** 1050 (1939).

⁶¹ D. Burk, R. J. Winkler and V. du Vigneaud *Proc. Am. Soc. Biol. Chem.* **1941** XX1.

⁶² F. E. Allison, S. R. Hoover and D. Burk *Science* **78** 217 (1933).

During vitamin H deficiency the tissues, for example, of chicks are practically devoid of this vitamin ⁶²

(b) *Physiological Action of Vitamin H*

The physiological action of vitamin H is not clearly understood. A close relationship between this vitamin and fat metabolism has been postulated. A certain increase in the fat and cholesterol synthesis in the liver of rats upon feeding vitamin H has been observed. This abnormal activity can be prevented by the addition of lipocane (an internal secretion of the pancreas) to the diet ⁶³

Biotin is made unavailable to the living organism (experiments with rats, chicks and yeast) by the formation of a complex with a protein constituent of raw egg white, and the typical syndromes of a biotin deficiency occur when, instead of the free biotin, the protein symplex is administered (This is the historically important egg white toxicity). The combination of biotin with the protein is stoichiometric and biotin cannot be recovered by dialysis. The protein which has the peculiar capacity of binding biotin is called 'avidin' and has been obtained in crystalline form ⁶⁴

13 AVITAMINOSIS

Rats develop peculiar and impressive skin changes on a diet deficient in vitamin H. These are accompanied by progressive emaciation and finally led to death. The skin lesions are differentiated from the pellagra or acrodermia syndrome since they are not predominantly a peripheral dermatosis ⁶⁵. The dermatitis can best be regarded as a seborrheic desquamative type. At the same time, biotin is involved to a certain extent in the maintenance of the normal pigment metabolism of the fur in rats and mice ⁶⁶. In human infants ⁶⁷ and in rats ⁶⁸ a deep brown pigmentation has been observed on the back. Male rats exhibit a greater sensitivity to vitamin H deficiency than female rats ⁶⁹. ⁶⁵ A high incidence of a special type of pneumonia which could be improved and even completely cured by the

⁶² R. E. Eakin, W. A. McKinley and R. J. Williams *Science* 92: 224 (1940)

⁶³ E. W. McHenry and G. Gavin *Proc. Am. Soc. Biol. Chem.* 1941: LXXXVII

⁶⁴ R. E. Eakin, E. E. Snell and R. J. Williams *J. Biol. Chem.* 136: 801 (1940) 140: 370 (1941)

⁶⁵ D. Pennington, E. E. Snell and R. E. Eakin *J. Am. Chem. Soc.* 64: 469 (1942)

⁶⁶ P. György *J. Biol. Chem.* 131: 733 (1939)

⁶⁷ P. György and C. E. Poling *Proc. Soc. Exptl. Biol. Med.* 45: 773 (1940)

⁶⁸ E. Moro *Ektrema Infantum und Dermatitis Seborrhoides* Berlin 1933, p. 7

⁶⁹ H. T. Parsons *J. Biol. Chem.* 90: 351 (1931)

⁶⁶ M. A. Boas *Biochem. J.* 21: 712 (1927)

administration of vitamin H⁶⁵ has been noted.⁷⁰ Certain disturbances of the nervous system in rats on a vitamin H deficient diet have been observed.⁷¹ Furthermore biotin deficiency in rats causes a typical denudation around the eyes. This syndrome is known as the 'spectacle eye condition'^{72a} and is prevented and cured by the administration of biotin.^{71b}

Vitamin H deficiency in chicks results in a specific dermatitis.^{71c} The bottoms of the feet become rough and calloused and in more severe cases encrusted and hemorrhagic cracks appear. In addition, mandibular lesions occur in the corners of the mouth and around the beak and the eye lids become swollen and stick together.

Experimental biotin deficiency in man on a diet in which about 30% of the total calories was supplied by desiccated egg white, resulted in the occurrence of a number of clinical symptoms similar to those of spontaneous avitaminosis. A striking ashy pallor of the skin and mucous membranes appeared, followed by an increasing dryness of the skin with marked reticulation and a fine branny desquamation. Extreme lassitude and somnolence, muscle pains, precordial distress and anorexia were observed. Parenteral administration of biotin cured these symptoms rapidly.^{71d}

In man vitamin H therapy has given curative results in a few isolated cases of acne vulgaris⁷³ and rosacea and of furunculosis. Certain cases of baldness in men are caused by seborrheic conditions and can be improved by vitamin H administration. More severe cases of seborrhea (overaction of the sebaceous glands) seem to be related to the skin disease called psoriasis which consists of an eruption of circumscribed rounded patches occurring chiefly on the elbows and knees, scalp and back. Encouraging results have been attained in these cases by administration of vitamin H.

⁶⁵ M. Gundel, I. György and W. P. Z. *Hyg. Infektionskrankh.* 113: 629 (1932).

⁷¹ C. M. Fendly and R. O. Stern, *Arch. Disease Childhood* 4: 1 (1929). J. G. Lea and T. J. Ryan and F. Kelly, *Biochem. J.* 31: 433 (1937).

⁷² J. Coldberger and R. D. Lill, *Pub. Health Rep. U. S. P. H. S.* 41: 102 (1926). A. Hourquand and H. C. Sherman, *J. Am. Chem. Soc.* 53: 7501 (1931). H. E. Robinson and R. C. Newton, *Abstracts Day of Biol. Chem. A. C. S. Kansas City* April 13-17 1936. S. Lepkovsky, T. H. Jukes and M. F. Krause, *J. Biol. Chem.* 115: 557 (1936). B. Sjollema, *Acta Biologica Neerland. Physiol. Pharmacol. Microbiol.* 102: 148 (1937). *Tsidschr. Dermatol.* 64: 986 (1937). P. K. River, I. Laszt and F. Verász, *Arch. exp. Physiol.* 239: 644 (1937). E. M. Mackay and R. H. Brown, *Proc. Soc. Exptl. Biol. Med.* 46: 713 (1941). J. J. Oleson, H. R. Bird, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.* 127: 23 (1939). J. J. Oleson, C. A. Elvehjem and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.* 43: 161 (1940). F. J. Pavcek and H. M. Baum, *Science* 93: 50 (1941).

⁷³ E. N. Olsen and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* 48: 349 (1941).

⁷⁴ D. M. Hegsted, J. J. Oleson, R. C. Mills, C. A. Elvehjem and F. B. Hart, *J. Nutrition* 20: 49 (1940). D. M. Hegsted, R. C. Mills, C. M. Briggs, C. A. Elvehjem and F. B. Hart, *Ibid.* 23: 175 (1942).

⁷⁵ V. P. Sydenhacker, S. A. Singal, A. P. Briggs, N. M. D. Vaughn and H. Isbell, *Science* 95: 176 (1942).

⁷⁶ W. M. H. J. *Invest. Dermatol.* 2: 205 (1939).

14 Requirements

Vitamin H is required by all animals investigated, such as rats, chicks⁷³ guinea pigs, rabbits, monkeys⁷⁴ dogs⁷ and man. Man needs 50 Rat Units of vitamin H when administered subcutaneously or about three to five times this amount when given *per os*.⁷⁶ Cattle do not necessarily need an external supply of vitamin H since the microorganisms in the rumen synthesize the vitamin.⁷⁷ The requirements of microorganisms (bacteria) have been discussed previously (see page 475).

Men have responded favorably to parenteral administration of 150–300 gamma of biotin per day. Chicks need about 2.5–10 gamma and rats about 1–3 gamma per day.

⁷³ A. T. Ringrose, L. C. Norris and G. F. Heuser, *Poultry Sci.* 10, 166 (1931).

⁷⁴ J. G. Leese, H. T. Parsons and E. Kelly, *Biochem. J.* 31, 433 (1937).

⁷⁵ P. György, *J. Biol. Chem.* 131, 733 (1939).

⁷⁶ P. György, *Ibid.* 119, XLIII (1937). F. Schultz, *Med. u. Chem. Abhandl. med. chem. Forschung stätten I. G. Farbenind.* 3, 143 (1936). P. György and C. S. Rose, *Proc. Soc. Exptl. Biol. Med.* 43, 73 (1940).

⁷⁷ M. I. Wegner, A. N. Booth, C. A. Elvehjem and E. B. Hart, *Proc. Soc. Exptl. Biol. Med.* 45, 789 (1940).

**THE GROUP OF
VITAMINS K**

THE GROUP OF VITAMINS K¹

1 Nomenclature and Survey

Names

Vitamins K²

Phylloquinones³ α β etc

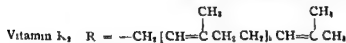
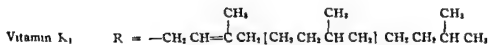
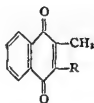
Coagulations Vitamin (German and Scandinavian term from which the present term vitamin K was derived)

Coagulation vitamin

Prothrombin factor

Antihemorrhagic vitamin

Structure



2 Chronology

- 1929 DAM⁴ observed subcutaneous and intramuscular hemorrhages in chicks raised on an artificial diet
- 1934 DAM and SCHÖNHEYDER⁵ concluded that the hemorrhagic conditions of the chicks were caused by an avitaminosis and called the new factor vitamin K.⁶

¹ H. R. Butt and I. A. M. Snell *Estams* A Phila delph 1941

² H. D. m. *Nat* e 135 652 (1935)

³ P. Karrer and A. Geiger *Helv Chim Acta* 22 945 (1939)

⁴ H. Dam *Biochim Z* 215 47 (1929) 220 158 (1930)

⁵ H. Dam and F. Schönheyder *Biochim J* 28 115 (1934)

⁶ H. Dam *Nat* e 135 657 (1935)

- 1937 DAM SCHÖNHEIDER and co worker⁷ found that vitamin K takes part in restoring and maintaining the normal prothrombin level of blood
- 1938 WARNER BRINKHOUS and SMITH⁸ BUTT SNELL and OSTERBERG⁹ and DAM and GLAVIND¹⁰ demonstrated clinically the usefulness of vitamin K for man
- 1939 DAM KARRER and co-workers¹¹ isolated pure vitamin K₁ and DOISY and co-workers¹² isolated pure vitamin K₂. The synthesis of vitamin K₁ was achieved independently in three different laboratories namely in those of ALMQUIST¹³ DOISY¹⁴ and FIESER¹⁵ ANSBACHER and FERNHOLZ¹⁶ discovered the anti hemorrhagic activity of 2 methyl 1,4 naphthoquinone
- 1940 DOISY and co workers¹⁷ elucidated the chemical structure of vitamin K

3 The Group of Vitamins K

The existence of only two naturally occurring vitamins K of high activity namely vitamins K₁ and K₂, has been proved with certainty. Phthiocol a naturally occurring compound, is also somewhat active but in considerably higher amounts. The inclusion of this compound into the class of naturally occurring vitamins K depends upon the proof that this compound is not an artefact from the alkaline hydrolysis procedure used for its isolation^{18, 19}. It is, however, conceivable that besides vitamins K₁ and K₂ other members of similar structure occur naturally, especially of the type with β unsaturated isoprenoid side chains in the 3 position of 2 methyl 1,4 naphthoquinone, such as 3 farnesyl or 3 geranyl derivatives²⁰.

Indication for the occurrence of at least one other factor has been obtained through the isolation of colorless fractions^{21, 22}. This factor is highly active and resembles in its behavior the oxides of vitamins K. It is, therefore, possible that this compound or compounds are oxides obtained during the procedure of isolation.

⁷ H Dam *Nature* 135 652 (1935) F Schönheider *Ibid* 135 505 (1935) H Dam F Schönheider and F Tage Hansen *Biochem J* 30 1075 (1936)

⁸ E D Warner K M Brinkhaus and H P Smith *Proc Soc Exptl Biol Med* 37 68 (1938)

⁹ H R Butt A M Snell and A J Osterberg *Proc Staff Meetings Mayo Clinic* 13 74 (1938)

¹⁰ H Dam and J Glavind *Lancet* 234 720 (1938) *Acta Med Scand* 96 108 (1938)

¹¹ H Dam A Geiger J Glavind P Karrer W Karrer F Rothchild and H Salomon *Helv Chim Acta* 22 310 (1939)

¹² R W McKee S B Binkley D W MacCorquodale S A Thayer and I A Doisy *J Am Chem Soc* 61 1295 (1939)

¹³ H J Almquist and A A Klose *Ibid* 61 557 (1939)

¹⁴ S B Binkley L C Cheney W L Holcomb R W McKee S A Thayer D W MacCorquodale and E A Doisy *Ibid* 61 2558 (1939)

¹⁵ L F Fieser *Ibid* 61 2559 (1939)

¹⁶ S Ansbacher and E Fernholz *Ibid* 61 1924 (1939)

¹⁷ S Binkley R W McKee S A Thayer and E A Doisy *J Biol Chem* 133 721 (1940)

¹⁸ L F Fieser *J Am Chem Soc* 61 2559 (1939)

¹⁹ L F Fieser *Ibid* 61 3467 (1939)

²⁰ L F Fieser M Tishler and W I Sampson *J Biol Chem* 137 659 (1941)

²¹ S Ansbacher E Fernholz and H B MacPhillamy *Proc Soc Exptl Biol Med* 42 65 (1939)

E Fernholz S Ansbacher and M I Moore *J Am Chem Soc* 61 1613 (1939)

4 Occurrence

Generally speaking vitamins K occur only in plants and in microorganisms. Which one of the group of vitamins K is present in each source is largely unknown but it is assumed that the green leaves of plants contain predominantly or solely vitamin K₁ whereas the microorganisms contain vitamin K₂.

The best sources of vitamin K₁ are the green leafy tissues of alfalfa, spinach, cabbage,³ ²⁴ kale, cauliflower, nettle, chestnut,⁵ etc. Vitamin K₁ is also present in tomatoes, hempseed, seaweed⁵ and soybean oil.²⁸ Fruits and cereals are very poor sources of the vitamin.

Vitamin K₂ occurs in many microorganisms, especially in most bacteria,⁷ while molds, yeasts and fungi contain no, or practically no, vitamin K. From a practical standpoint it is important that the microorganisms in the intestinal tract contain abundant quantities. Most putrefied animal and plant materials contain high amounts due to bacterial growth.

Animal materials contain very little vitamin K, although milk and eggs contain small amounts. Hog liver²⁸ is the richest animal source found so far. The livers of chicks²⁸ ²⁹ ³⁰ and of rats²⁸ contain very little vitamin K.

5 Isolation

The isolation of vitamin K₁ from, for example, alfalfa leaf meal is carried out by first extracting the dried plant material with petroleum ether, hexane³¹ or acetone³² followed by an adsorption of the chlorophyll present on zinc carbonate³³, magnesium oxide³⁴ or phosphotungstic acid.³⁴ Upon cooling the remaining solution some material precipitates and is discarded. The solution is then subjected to fractional distillation.³ ³⁵ Vitamin K₁ distills between 120° and 140° C at 10⁻⁴ mm Hg. Several compounds, especially sterols, are separated from the distillate by crystallization from

³ H. Dam, *Nature* 135: 65 (1935); *Biochem. J.* 29: 171 (1935).

²⁴ H. J. Almquist and E. L. R. Stokstad, *Nature* 136: 31 (1935); *J. Biol. Chem.* 111: 10 (1935).

²⁸ H. Dam and J. Glavin, *Biochem. J.* 32: 48 (1938).

²⁹ H. J. Almquist and E. L. R. Stokstad, *J. Nutrition* 14: 235 (1937).

³⁰ H. J. Almquist, C. F. Pentler and E. Mecchi, *Proc. Soc. Exptl. Biol. Med.* 38: 336 (1938).

³¹ H. Dam and I. Schönheyder, *Biochem. J.* 30: 897 (1936).

³² H. Dam and J. Glavin, *Lancet* 234: 720 (1938).

³³ H. J. Almquist and E. L. R. Stokstad, *J. Nutrition* 12: 379 (1936).

³⁴ H. J. Almquist and E. L. R. Stokstad, *Ibid.* 12: 329 (1936).

³⁵ H. Dam and I. Schönheyder, *Biochem. J.* 30: 897 (1936).

³⁶ P. Karrer, A. Ceiger, R. Legler, A. Rüegger and H. Salomon, *Helv. Chim. Acta* 22: 1464 (1939).

³⁷ A. A. Klose and H. J. Almquist, *J. Am. Chem. Soc.* 61: 53 (1939).

³⁸ B. Rieg, C. I. Schweitzer and P. C. Smith, *J. Biol. Chem.* 129: 405 (1939).

³⁹ H. J. Almquist, *Ibid.* 120: 63 (1937); *Helv. Chim. Acta* 20: 31 (1937).

acetone By application of the chromatographic adsorption technique using first dehydrated magnesium sulfate and then zinc carbonate the pure vitamin can be obtained³³ Other adsorbing agents such as calcium sulfate,³⁷ weakly acidic fuller's earth (Florex)³⁸ Permutit,³⁹ 40 Darco⁴⁰ etc have been used for the isolation of both vitamins K₁ and K₂

The isolation of vitamin K according to these procedures is relatively difficult A very simple method has however been devised for the isolation of vitamin K in the reduced form⁴¹ This consists in reducing a crude vitamin K concentrate in alcohol solution with sodium hydrosulfite and extracting the reduced vitamin with petroleum ether The solvent solution is then extracted with 5% sodium hydroxide containing hydrosulfite which removes considerable amounts of foreign matter The petroleum ether solution is then extracted with Claisen's alkali solution⁴² prepared from potassium hydroxide, water and methanol, to which hydrosulfite has been added The alkaline solution is diluted with water and the reduced vitamin is extracted with ether The ether solution is washed with hydrosulfite solution and the reduced vitamin is obtained by evaporation of the solvent The vitamin itself is obtained from the reduced compound by shaking in ether solution with silver oxide and magnesium sulfate

Vitamin K can also be isolated in the form of the diacetate of the reduced form which is prepared by dissolving the vitamin in acetic anhydride and adding zinc dust and pyridine⁴³ 44

6 Properties

The vitamins K are soluble in most of the common organic solvents, especially in ether, petroleum ether, hexane acetone, etc but are insoluble in water and only sparingly soluble in methanol The vitamins are essentially thermostable⁴⁵ They are very sensitive to alkali⁴⁶ and to light from various sources, such as sunlight,⁴⁷ 48 the illumination from ordinary

³³ H Dam *Z Vitaminforsch* 8 248 (1938 '39)

³⁴ B Riegel C E Schweitzer and P C Smith *J Biol Chem* 129 494 (1939)

³⁵ S B Binkley D W MacCorquodale S A Thayer and E A Doisy *Ibid* 130 219 (1939)

³⁶ R W McKee S B Binkley S A Thayer D W MacCorquodale and E A Doisy *Ibid* 131 127 (1939)

³⁷ L F Fieser *J Am Chem Soc* 61 3467 (1939)

³⁸ L Claisen *Ann* 418 96 (1919)

³⁹ L F Fieser *J Am Chem Soc* 61 3467 (1939)

⁴⁰ S B Binkley D W MacCorquodale I C Cheney S A Thayer R W McKee and E A Doisy *Ibid* 61 1612 (1939)

⁴¹ H J Almquist *J Biol Chem* 120 635 (1937)

⁴² H J Almquist and F I R Stokstad *J Nutrition* 14 23 (1937)

⁴³ D W MacCorquodale S B Binkley R W McKee S A Thayer and E A Doisy *Proc Soc Exptl Biol Med* 40 482 (1939)

⁴⁴ P Karrer and A Geiger *Helv Chim Acta* 22 94 (1939)

Mazda light bulbs⁴⁷ and ultraviolet light⁴⁸ The vitamins K exhibit typical absorption characteristics in the ultraviolet region with maxima at 243 248 261 270 and 328 m μ (Fig 24)

Vitamin K₁ is a yellow oil which melts at about -20° C The redox potential is $E_m = +0.005$ volt Vitamin K₁ exhibits a white fluorescence in the light of an argon lamp⁵⁰ This phenomenon has not been observed in simple naphthoquinones without side chains in the 3 position

Vitamin K₂ is a yellow crystalline solid m p 53.5–54.5 $^{\circ}$ C⁵¹

2-Methyl-1,4-naphthoquinone is a lemon yellow crystalline powder with faint but characteristic odor melting at 106 $^{\circ}$ C It is soluble in

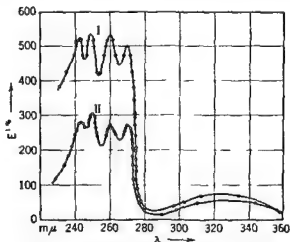


Fig 24—Absorption spectra of vitamin K₁ (I) and of vitamin K₂ (II) (D T Ewing J M Vandenbelt and O Kamin)

water to the extent of 0.13 mg per ml The absorption spectrum of 1,4-naphthoquinone and the diacetate of the hydroquinone are shown in Fig 25

7 Chemical Constitution

(a) The Constitution of Vitamin K₁

Vitamin K₁ has the empirical formula C₃₁H₄₆O₂ and, according to potentiometric titrations with sodium hydrosulfite a molecular weight of 450⁵² The presence of the oxygen atoms as quinone oxygens was deduced from the redox potential which proved to be very similar to that

⁴⁷ H J Almquist *J Biol Chem* 117 517 (1937)

⁴⁸ H J Almquist and A A Klotz *J Am Chem Soc* 61 7 (1939)

⁴⁹ B Brinkley R W McPhee S A Thayer and L A Dossy *ibid* 133 71 (1940)

⁵⁰ P Karrer and A Geiger *Helv Chim Acta* 22 94 (1939)

of many 1,4 quinones such as, for example, the anthraquinone hydroanthraquinone system⁵⁰ The presence of a quinoid structure was also suspected from the absorption spectrum of the vitamin⁵¹ which proved to be very similar to 2,3 disubstituted 1,4 naphthoquinones Further indications were the instability of the vitamin toward alkali and light and finally the absorption of eight atoms of hydrogen upon catalytic hydrogenation⁵ ($I \rightarrow II$) The reduced compound is colorless, but upon exposure to light it is oxidized to a yellow compound (III) the color of which is similar to that of the vitamin itself This suggests that the original

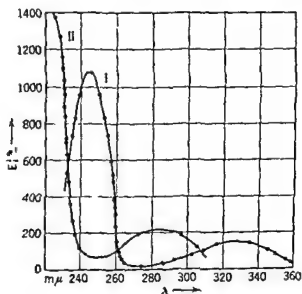
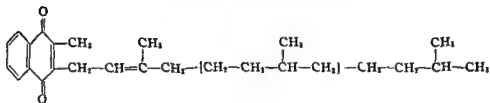


Fig. 25—Absorption spectra of 1,4-naphthoquinone (I) and the diacetate of 1,4-naphthohydroquinone (II) (D. T. Fwing, J. M. Vandenberg, and O. Kamm)

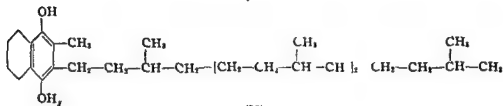
vitamin is a quinone which is reduced catalytically and partially reoxidized. Further proof was furnished by reductive acetylation which yielded a crystalline diacetate (IV) (formula on page 488) from which the vitamin could be recovered by hydrolysis by means of a Grignard reaction, followed by air oxidation⁵² The color of the quinone is yellow, which suggests a 1,4 position of the oxygen atoms, since the only other possible quinones namely the 1,2 quinones, are red.

⁵⁰ R. W. McKee, B. Binkley, D. W. MacCorquodale, S. A. Thayer and F. A. Dowsy, *J. Am. Chem. Soc.* 61, 1293 (1939).

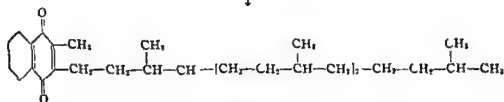
⁵¹ S. B. Binkley, D. W. MacCorquodale, I. C. Cheney, S. A. Thayer, R. W. McKee and F. A. Dowsy, *Ibid.* 61, 1621 (1939).



(I)



(II)



(III)

Vitamin K_1 upon oxidation with an excess of chromic acid yielded phthalic acid (V)⁵⁵ This shows that the aromatic non quinoid ring has no side chains attached and suggests that a 1,4 naphthoquinone is present which then must be substituted in the 2 and 3 positions. Mild chromic acid oxidation yielded 2 methyl 1,4 naphthoquinone 3 acetic acid (VI)⁵⁶ which was identified by comparison with a synthetic specimen.⁵⁶ Similarly chromic acid oxidation of the diacetate of dihydro vitamin K_1 yielded 1,4 diacetoxy 2 methyl naphthalene 3 acetic acid,⁵⁶ the methyl ester of which proved to be identical with the synthetic compound of the indicated structure.

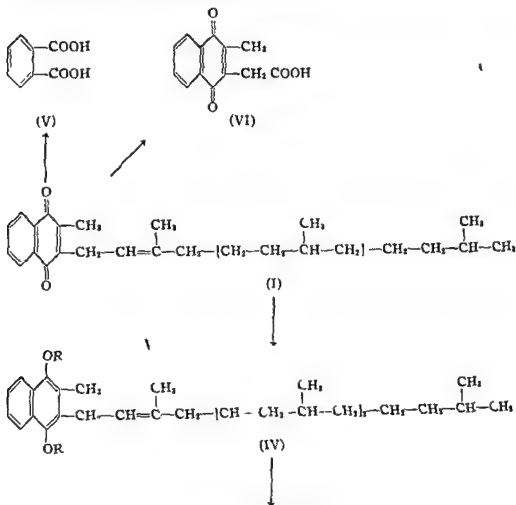
As indicated above catalytic hydrogenation of vitamin K_1 yielded a hydroquinone by absorption of four mols of hydrogen. Since hydrogenation

⁵⁵ D. W. McCorquodale, S. B. Buckley, S. A. Thayer, and A. Day, *J. Am. Chem. Soc.* 61, 1938 (1939).

⁵⁶ S. B. Buckley, I. C. Chen, J. W. F. Hulcomb, R. W. McKee, S. A. Thayer, D. W. McCorquodale, and F. A. Doyle, *Ibid.* 61, 2558 (1939).

tion of a naphthoquinone nucleus would require only three mols, the presence of a double bond in a side chain was indicated. This was proved by oxidation of the diacetate of dihydro vitamin K₁ with ozone which yielded a ketone, C₁₈H₃₆O₈ (VIII) which was found to be identical⁸⁶ with the corresponding ketone obtained by oxidation of phytol⁸⁷. The constitution of this ketone is 2,6,10 trimethyl pentadecanone 14. The other reaction product from the ozonolysis is 1,4 diacetoxy 2 methyl naphthalene 3 acetaldehyde (VII) which was characterized as the semicarbazone^{88, 89}.

These degradation reactions prove conclusively the structure of vitamin K₁ as 2 methyl 3 phytyl 1,4 naphthoquinone.

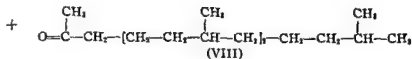
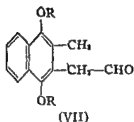


(Formula continued on following page)

⁸⁶ F. G. Fischer and K. Löwenberg *Ann.* 464 69 (1923)

⁸⁷ B. Binkley, R. W. McKee, S. A. Thayer and F. A. Dowsy *Proc. Am. Soc. Biol. Chem.* 1940 XII

⁸⁸ B. Binkley, R. W. McKee, S. A. Thayer and F. A. Dowsy *J. Biol. Chem.* 133, 721 (1940)



(b) *The Constitution of Vitamin K₂*

The methods used for the elucidation of the structure of vitamin K₁ and the knowledge gained from the proof of the structure of this vitamin were successfully applied for the determination of vitamin K₂.

Vitamin K₂ has the empirical formula C₄₁H₅₆O₂. Upon reductive acetylation a diacetate of dihydro vitamin K₂ is formed⁴⁰ (I → II) which proves the presence of the two oxygens in a quinone structure. The quinone structure is also revealed by the absorption spectrum, which is very similar to that of vitamin K₁. (See Fig. 24 on page 485.)

Upon catalytic hydrogenation vitamin K₂ absorbs 9 mols of hydrogen.⁴¹ Since three mols are required for reduction of the quinone structure, the presence of six double bonds in the side chains is indicated. This assumption is confirmed by the fact that dihydro vitamin K₂ diacetate adds 12 atoms of bromine. These six double bonds are not in conjugation to each other since no addition product is formed with maleic anhydride and since the ultraviolet absorption spectrum does not indicate the presence of any conjugated double bonds in addition to the quinone structure.

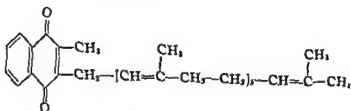
Oxidation of vitamin K₂ with ozone⁴² yields 1,4 diacetoxy 2-methyl acetaldehyde (III) which was characterized as the semicarbazone. Levulin aldehyde (IV) was further isolated from the oxidation product and identified as the bis-2,4-dinitro phenyl hydrazone. Assuming that 1 mol of vitamin K₂ would yield 5 mols of levulin aldehyde, this compound was obtained in an 81% yield. Acetone (V) was obtained also from the ozonolysis in the form of its 2,4-dinitro phenyl hydrazone in a yield of 56% assuming that one mol of acetone originates from one mol of the vitamin.

⁴⁰ S. B. Buckley, D. W. MacCorquodale, I. C. Chaffey, S. A. Thayer, R. W. McKee and E. A. Dossy, *J. Am. Chem. Soc.* 61, 1612 (1939).

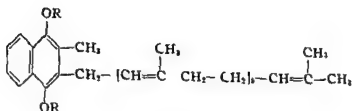
⁴¹ R. W. McKee, S. B. Buckley, D. W. MacCorquodale, S. A. Thayer and E. A. Dossy, *ibid.* 61, 1293 (1939).

⁴² S. B. Buckley, R. W. McKee, S. A. Thayer and E. A. Dossy, *Proc. Am. Soc. Biol. Chem.* 1940, XII.

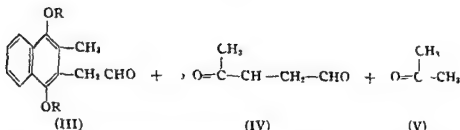
⁴³ S. B. Buckley, R. W. McKee, S. A. Thayer and E. A. Dossy, *J. Biol. Chem.* 133, 721 (1940).



(I)



(II)



(III)

(IV)

(V)

On the basis of these degradation products vitamin K₂ has the constitution (I) of a 2-methyl-3-bis(farnesyl)naphthoquinone 1,4⁶⁴

8 Synthesis

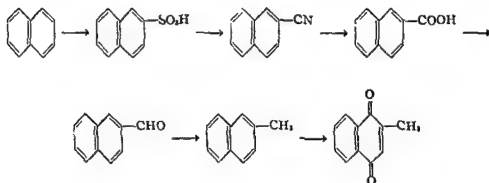
(a) Synthesis of 2-Methyl-1,4-naphthoquinone

The simplest method for the synthesis of 2-methyl-1,4-naphthoquinone is the oxidation of 2-methyl-naphthalene. The latter compound is a by-product obtained from the coal tar industry.⁶⁵ The oxidation is best accomplished by means of chromic acid in acetic acid⁶⁶⁻⁶⁷ at temperatures

⁶⁴ S. B. Bakley, R. W. McKee, S. A. Ihayr and F. A. Dossy, *J. Biol. Chem.* **133**, 91 (1940).
⁶⁵ I. Karrer and A. Lipprecht, *Helv. Chim. Acta* **23**, 272 (1940).
⁶⁶ E. A. Coulson, *J. Soc. Chem. Ind.* **60**, 123 (1941).
⁶⁷ K. Irie and W. Fohmann, *Ber.* **54**, 2918 (1921).
⁶⁸ R. J. Anderson and M. S. Newman, *J. Biol. Chem.* **103**, 406 (1933).

below 50°C ^{68, 69} This oxidation can however also be carried out by other oxidizing agents such as molecular oxygen (air), hydrogen peroxide⁷⁰ etc Yields between 30% and 60% of theory have been reported for this oxidation

2 Methyl 1,4 naphthoquinone has also been synthesized by a simple and practical reaction sequence from naphthalene⁷¹ Naphthalene is sulfonated in the β position and the sodium salt yields β naphthoic acid nitrile upon treatment with $\text{K}_4\text{Fe}(\text{CN})_6$ The nitrile is hydrolyzed to the free β naphthoic acid the Ba salt of which gives upon distillation with barium formate the corresponding aldehyde in 60% yield Clemmensen reduction of the oxo compound yields 2 methyl naphthalene which is converted by oxidation into 2 methyl naphthoquinone



A total synthesis of 2 methyl 1 4 naphthoquinone has been accomplished⁷² by condensing benzene by means of aluminum chloride with the anhydride of methyl succinic acid which is obtained from either citric acid or *d* tartaric acid The reaction product α methyl β benzoyl propionic acid, is reduced by the Clemmensen method to yield α methyl γ phenyl butyric acid Ring closure of its chloride with AlCl_3 yields 2 methyl α tetralone which is converted into 2 methyl 1 2,3 4-tetrahydro naphthalene by reduction Dehydrogenation with sulfur or with selenium yields 2 methyl naphthalene which is oxidized to the quinone

⁶⁸ L I Smith and I M Webster *J Am Chem Soc* 59 60 (1937)

⁶⁹ L F Fieser W P Campbell E M Frey and M D Gies *ibid* 61 539 3216 (1939)

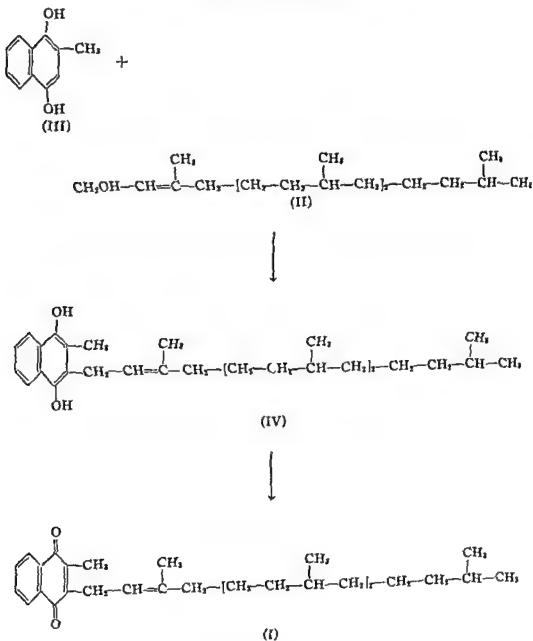
⁷⁰ R T Arnold and R Larson *J Org Chem* 3 900 (1940)

⁷¹ P P T Sah W B Gill and H Holten *Ber* 73 702 (1940) 1 1 T Sah *Rec trav chim* 59 161 (1940)

⁷² P P T Sah and W Brüll *Ber* 73 1430 (1940)

(b) *Synthesis of Vitamin K₁*

The synthesis of vitamin K₁ (I) has been carried out from 2 methyl 1,4 naphthoquinone and phytol (II) by a number of different methods. Thus the synthesis has been effected from these compounds alone,⁷¹ or, better, from 2 methyl 1,4 naphthohydroquinone (III) and phytol in the



presence of catalysts, such as oxalic acid or trichloro acetic acid⁷⁴ using dioxane as a solvent. Another variation is to use the mono sodium salt of 2 methyl 1,4 hydroquinone and phytyl bromide⁷⁵. The hydroquinone has also been condensed with phytol in benzene solution using zinc chloride as the condensing agent. Vitamin K₁ hydroquinone (IV) is obtained from the methods involving the use of the hydroquinone and is then oxidized to the vitamin (I) by air or silver oxide.

The best yields can be obtained by working under mildly acidic conditions. In the presence of strong acids such as mineral acids, the condensation is carried further giving rise to compounds of the vitamin E type. Condensation in alkaline medium, on the other hand, gives low yield and undesirable dark brown reaction mixtures from which it is difficult to isolate the pure vitamin K₁.

Vitamin K₁ has also been synthesized in low yields from phthiocol in its reduced form, and phytol⁷⁶.

(c) *Synthesis of Vitamin K₂*

The synthesis of vitamin K₂ has not been accomplished as yet.

9 Industrial Methods of Preparation

The concentrates of natural vitamins K such as alfalfa concentrates are still of considerable industrial interest, since they contain the natural vitamin K₁ which is clinically used. Synthetic vitamin K₁ is available, prepared according to the methods previously described but is not widely used due to the high cost. The synthetic 2 methyl naphthoquinone, which is at least as active although at high doses somewhat more toxic than the natural vitamins K, is offered on the market and is used clinically.

Water soluble forms of vitamin K have been introduced on the market. While the natural vitamins and 2 methyl naphthoquinone are fat soluble and require the presence of bile acids for proper absorption from the intestinal tract the water soluble forms are absorbed as such and are also important for intravenous administration. The following water soluble compounds of vitamin K action are used. 2 methyl 1,4 naphtho

⁷⁴ L. F. Fieser *J. Am. Chem. Soc.* 61 2359 (1939).

⁷⁵ C. B. B. Kley, I. C. Cheney, W. F. Holcomb, R. W. McKee, S. A. Thayer, D. W. MacCorquodale and E. A. Doty *J. Biol. Chem.* 61 2558 (1939). D. W. MacCorquodale, I. C. Cheney, S. B. Bakley, W. F. Holcomb, R. W. McKee, S. A. Thayer and E. A. Doty *J. Biol. Chem.* 131 337 (1939).

⁷⁶ M. T. Hill, L. F. Fieser and R. I. W. Noller *J. Am. Chem. Soc.* 62 1987 (1940).

hydroquinone 3 sodium sulfonate,⁷⁷ 4 amino, 2 methyl 1 naphthol hydrochloride^{78 79 80} and sodium 2 methyl 1,4 naphthohydroquinone diphosphate^{81 82 83}

10 Biogenesis

The vitamins K are synthesized by plants and by certain microorganisms. In plants, the site of synthesis is in the green leaves. Thus the tops of carrots contain a considerable amount of vitamin K while the roots contain practically none.⁸⁴ The synthesis is greatly influenced by sunlight. Peas grown in the dark contain only small amounts while control plants raised in light contain considerably more.⁸⁵ The outer leaves of cabbage contain about four times more vitamin K than the inner leaves.⁸⁶

The synthesis of vitamin K₁ is presumably accomplished by methods similar to those used in the laboratory, that is, using phytol and methyl naphthoquinone as building units.⁸⁶ Vitamin K₂ is synthesized in certain microorganisms which live on vitamin K free diets.⁸⁷ *Coli* bacteria synthesize vitamin K on a synthetic medium which contains as organic constituents only glucose, citrate and asparagin.⁸⁸

11 Specificity

The physiological activity of the vitamins K₁ and K₂ is by no means restricted to these compounds. A number of other similar compounds have the same property. From the vast amount of work done to elucidate the specificity of the vitamin K action the following conclusions can be drawn. The quinones may be converted into the corresponding hydroquinones, their esters and ethers without essential loss of efficacy as long as the derivatives can be reconverted into the quinones under more or less simple conditions which also may occur in the organism. The unsub-

⁷⁷ M. B. Moore and I. J. Kirchmeyer quoted in H. R. Butt and A. M. Snell *Vitamin A*, Philadelphia 1941. M. B. Moore *J. Am. Chem. Soc.* **63**, 2049 (1941).

⁷⁸ E. A. Dossy, D. W. MacCorquodale, S. A. Thayer, S. B. Binkley and R. W. McKee *Science* **90**, 407 (1939).

⁷⁹ H. J. Almquist and A. A. Klose *Proc. Soc. Exptl. Biol. Med.* **43**, 55 (1940).

⁸⁰ D. Richtert, S. A. Thayer, R. W. McKee, S. B. Binkley and E. A. Dossy *Ibid.* **44**, 601 (1940).

⁸¹ L. F. Fieser and I. M. Frey *J. Am. Chem. Soc.* **62**, 228 (1940).

⁸² R. H. K. Foster, J. Lee and U. V. Solmsen *Ibid.* **62**, 453 (1940).

⁸³ S. Ansbacher, E. Fernholz and M. A. Dolliver *Proc. Soc. Exptl. Biol. Med.* **43**, 65 (1940).

⁸⁴ H. J. Almquist *J. Biol. Chem.* **117**, 517 (1937).

⁸⁵ H. Dam and J. Clavind *Biochem. J.* **32**, 483 (1938).

⁸⁶ L. F. Fieser, W. P. Campbell and E. M. Frey *J. Am. Chem. Soc.* **61**, 2206 (1939).

⁸⁷ H. J. Almquist, C. F. Pentler and E. Mecchi *Proc. Soc. Exptl. Biol. Med.* **38**, 336 (1938). A. I. terberg *Proc. Staff Meetings Mayo Clinic* **13**, 72 (1938).

⁸⁸ H. D. M. J. Clavind, S. Orla Jensen and A. D. Orla Jensen *Naturwissenschaften* **29**, 287 (1941).

stituted benzene ring of the vitamin molecule cannot be substituted without loss of activity. The 2 methyl group cannot be replaced by hydrogen or higher alkyl groups without considerable decrease in activity. The substituents on the 3 position, however, can vary considerably and the change in efficacy is only slight. The most remarkable fact is that when the substituent in 3 position is hydrogen, that is when 2 methyl 1,4 naphthoquinone is tested an activity of from two to four times that of vitamin K₁ per weight unit is observed. This unique activity of 2 methyl naphthoquinone has caused widespread interest which led to the introduction of this compound into clinical therapy. Its unusual efficacy has been explained by the hypothesis that this compound itself does not act as a vitamin in the organism but that it is converted in the organism into a quinone of the true vitamin K type. On the other hand it has been postulated that the vitamins K₁ and K₂ owe their activity to their degradation in the organism to 2 methyl 1,4 naphthoquinone.⁵¹ While the latter hypothesis appears less attractive, no experimental proof for the validity of the former can be offered other than the fact that such a synthesis is easily accomplished in the laboratory and that the building units for a side chain of the vitamin K₁ or K₂ type are readily available.

The effect of variations of the side chain in 3 position are noteworthy. A double bond in the β γ position contributes to the potency while unsaturation at points more remote from the quinoid nucleus is without influence. A branched side chain built from isoprene units is more active than a strictly straight chain and maximum activity is reached when some 20 or 30 carbon atoms are present.

Finally the fact that the activity of the 2,3-oxides of the vitamins K is almost equivalent to that of the vitamins is noteworthy. Compounds which may be identical with these have been isolated from natural materials but their identity has not been established as yet and the possibility of an artefact during the process of isolation cannot be ruled out.

These conclusions have been drawn from a large number of experiments with many compounds. In the following tables comparative data are given for the minimum effective dose of various series of compounds which are chemically closely related. The effective dose in these tables is defined as the minimum amount of material which, when administered in 0.1 cc. of peanut oil, will reduce the blood clotting times of 60 to 80% of vitamin K-deficient chicks to less than 10 minutes during an 18 hour period.⁵²

⁵¹ H. J. Almquist, *Physiol. Rev.* 21, 194 (1941).

⁵² I. F. Flier, M. Tibbitts and W. L. Sampson, *J. Biol. Chem.* 137, 653 (1941).

TABLE I⁹¹
2 METHYL-3 ALKYL AND 3 β ALKENYL 1 4 NAPHTHOQUINONES

Compound	Effective dose in γ
2 Methyl 3 phtyl 1 4 naphthoquinone ^{91 92} (Vitamin K ₁)	1
2 Methyl 3 difarnesyl 1 4 naphthoquinone ^{93 94} (Vitamin K ₂)	1 6
2 Methyl 3 farnesyl 1 4 naphthoquinone ^{95 96}	5
2 Methyl 3 (β γ dihydrophytyl) 1 4 naphthoquinone ^{96 97 98}	8
2 Methyl 3 geranyl 1 4 naphthoquinone ⁹²	25
2 Methyl-3 cinnamyl 1 4 naphthoquinone ⁹⁹	25
2 Methyl 3 (β γ γ trimethyl allyl) 1 4 naphthoquinone ⁹⁹	50
2 3 Dimethyl 1 4 naphthoquinone ¹⁰⁰	50
2 Methyl-3 benzyl 1 4 naphthoquinone ⁹⁹	200
2 Methyl 3 hydrocinnamyl 1 4-naphthoquinone ⁹⁷	300
2 Methyl 3 octadecyl 1 4 naphthoquinone ^{101 100}	Inactive at 1000

TABLE II¹⁰¹
2 ALKYL AND 2 β ALKENYL 1 4 NAPHTHOQUINONES

Compound	Effective dose in γ
2 Methyl 1 4 naphthoquinone ¹⁰²	0 3
2 Phtyl 1 4 naphthoquinone ^{95 103 104}	50
2 Farnesyl 1 4 naphthoquinone ^{96 103}	500
2 (β γ Dihydro phtyl) 1 4 naphthoquinone	600
2 <i>n</i> Hexadecyl 1 4 naphthoquinone ¹⁰⁵	More than 600
2 <i>n</i> Octadecyl 1 4 naphthoquinone ¹⁰⁵	More than 600
2 Allyl 1 4 naphthoquinone ^{106 107}	800
2 Geranyl 1 4 naphthoquinone ^{96 103}	1000
2 Ethyl 1 4 naphthoquinone ^{106 108 109}	Inactive at 1000
2 <i>n</i> Propyl 1 4 naphthoquinone ^{105 108 109}	Inactive at 1000

⁹¹ L F Fieser *J Am Chem Soc* 61 259 (1939)

⁹² L F Fieser *Ibid* 61 3467 (1939)

⁹³ R W McKee & B Binkley D W MacCorquodale & A Thayer and E A Dossy *Ibid* 61 1295 (1939)

⁹⁴ H Dam J Glavind and P Karrer *Helv Chim Acta* 23 224 (1930)

⁹⁵ L F Fieser M Tishler and W I Sampson *J Am Chem Soc* 62 93f 1f-8 (1940)

⁹⁶ L F Fieser M Tishler and N L Wendler *Ibid* 62 7861 (1940)

⁹⁷ M Tishler L F Fieser and N L Wendler *Ibid* 62 2866 (1940)

⁹⁸ P Karrer and A Epprecht *Helv Chim Acta* 23 272 (1940)

⁹⁹ L F Fieser W P Campbell E M Frey and M D Gates *J Am Chem Soc* 61 3253 3216 (1939)

¹⁰⁰ E Fernholz S Ansbacher and H B MacPhillamy *Ibid* 62 430 (1940)

¹⁰¹ L F Fieser M Tishler and W L Sampson *J Biol Chem* 137 659 (1941)

¹⁰² S Ansbacher and E Fernholz *J Am Chem Soc* 61 1974 (1939)

¹⁰³ L F Fieser M Tishler and W L Sampson *Ibid* 62 996 1628 (1940)

¹⁰⁴ H Dam J Glavind and P Karrer *Helv Chim Acta* 23 224 (1940)

¹⁰⁵ E Fernholz S Ansbacher and H B MacPhillamy *J Am Chem Soc* 62 430 (1940)

¹⁰⁶ L F Fieser W P Campbell and F M Frey *Ibid* 61 2206 (1939)

¹⁰⁷ L F Fieser D M Bowen W P Campbell M Fieser E M Frey R N Jones B Riegel C E Schweitzer and P G Smith *Ibid* 61 1970 (1939)

¹⁰⁸ M Tishler and W I Sampson *Ibid* 61 563 (1939)

¹⁰⁹ B Sjogren *Z physiol Chem* 264 1 (1939)

TABLE III¹¹⁰
 HIGHLY ALKYLATED NAPHTHOQUINONES

Compound	Effective dose in γ
2 Ethyl 3 phetyl 1 4 naphthoquinone ¹¹¹ ¹¹²	1000
2 3 Diallyl 1 4 naphthoquinone ¹¹³ ¹¹⁴	1000
2 5 Dimethyl 1 4 naphthoquinone ¹¹⁵	500
2 6 Dimethyl 1 4 naphthoquinone	Inactive at 1000
2 7 Dimethyl 1 4 naphthoquinone	1000
2 8 Dimethyl 1 4 naphthoquinone ¹¹⁶	500
6 7 Dimethyl 1 4 naphthoquinone ¹¹³ ¹¹⁴	Inactive at 1000
2 6 Dimethyl 3 phetyl 1 4 naphthoquinone ¹¹⁶	Inactive at 1000
1 1 Dimethyl 3 <i>tert</i> butyl 1 4-dihydro anthraquinone ¹¹⁷	Inactive at 1000
2 (δ Methyl γ pentenyl) 1 4 dihydro anthraquinone ¹¹⁷	Inactive at 1000
1 2 4 Trihydroxy anthraquinone ¹¹⁸	100

 TABLE IV¹¹⁹
 CARBETHOXY AND HYDROXY NAPHTHOQUINONES

Compound	Effective dose in γ
3 Methyl-3-carbethoxy 1 4 naphthohydroquinone ¹²⁰	25
2 Methyl 5 hydroxy 1 4 naphthoquinone ¹²¹	400
2 Methyl-3 hydroxy 1 4 naphthoquinone (Phthiocol) ¹²²	500
5-Hydroxy 1 4 naphthoquinone (Juglone) ¹²³	Inactive at 1000 feebly active at 10 mg
2 Hydroxy 1 4 naphthoquinone (Lawsone) ¹²⁴ ¹²⁵	Inactive at 1000 active at 10 mg
2 Hydroxy 3 dimethyl allyl 1 4 naphthoquinone (I apachol) ¹²⁶	Inactive at 1000 active at 5 mg
2 β Heptenyl 3 hydroxy 1 4 naphthoquinone	Inactive at 1000
2 Farnesyl-3 hydroxy 1 4 naphthoquinone ¹²⁶	Inactive at 1000
2 Methyl 3 (γ hydroxy-dihydrophytyl) 1 4 naphthoquinone ¹²⁶	Inactive at 1000
Hydroquinone-diacetate ¹²⁶	Inactive at 1000

¹¹⁰ I. F. Fieser, M. Tishler and W. I. Sampson, *J. Biol. Chem.* **137**, 650 (1941)

¹¹¹ I. F. Fieser, *J. Am. Chem. Soc.* **61**, 259 (1939)

¹¹² I. F. Fieser, *ibid.* **61**, 3467 (1939)

¹¹³ I. F. Fieser, W. P. Campbell and F. M. Fyfe, *ibid.* **61**, 2706 (1939)

¹¹⁴ I. F. Fieser, D. M. Bowen, W. P. Campbell, F. M. Frey and M. D. Gates, *ibid.* **61**, 196 (1939)

¹¹⁵ M. Tishler, I. F. Fieser and N. I. Wendler, *ibid.* **62**, 866 (1940)

¹¹⁶ I. F. Fieser, *ibid.* **61**, 3467 (1939)

¹¹⁷ I. F. Fieser and C. W. Wiegand, *ibid.* **62**, 153 (1940)

¹¹⁸ G. J. Martin and C. F. Irsch, *J. Biol. Chem.* **137**, 169 (1941)

¹¹⁹ I. F. Fieser, M. Tishler and W. I. Sampson, *ibid.* **137**, 69 (1941)

¹²⁰ C. F. Koelsch and D. J. Byers, *J. Am. Chem. Soc.* **62**, 560 (1940)

¹²¹ I. F. Fieser and J. T. Dunn, *ibid.* **58**, 574 (1936)

¹²² H. J. Almquist and A. A. Klose, *ibid.* **61**, 1611 (1939)

¹²³ R. Kuhn, K. Walle, F. Weygand, T. Moll and I. H. Pöschel, *Verh. Ges. Naturforsch. 27*, 518 (1939)

¹²⁴ H. Dam, J. Chaud and P. K. Frey, *Helv. Chim. Acta* **23**, 24 (1940)

¹²⁵ M. Tishler, I. F. Fieser and N. I. Wendler, *J. Am. Chem. Soc.* **62**, 7866 (1940)

¹²⁶ M. Tishler, I. F. Fieser and N. I. Wendler, *ibid.* **62**, 108 (1940)

TABLE V¹²⁷
NAPHTHOQUINONE OXIDES

Compound	Effective dose in γ
Vitamin K ₁ oxide ^{128 129}	1 2
2 Methyl 1 4-naphthoquinone oxide ^{130 131}	5
2,3 Dimethyl 1 4 naphthoquinone oxide ^{128 129}	25
2 Methyl-3-cinnamyl 1 4 naphthoquinone oxide ^{128 129}	150
2 Phytyl 1 4 naphthoquinone oxide ^{128 129}	200
2 Farnesyl 1 4 naphthoquinone oxide ^{128 129}	1000
2 7 Dimethyl 1 4 naphthoquinone oxide ¹³⁰	Inactive at 1000

TABLE VI¹³²
MISCELLANEOUS QUINONES

Compound	Effective dose in γ
2 3 5-Trimethyl 1 4 benzoquinone	Inactive at 1000
Duroquinone ¹³³	10000
2 3 5 Trimethyl 6 phytyl 1 4 benzoquinone ^{134 135}	Inactive at 1000
α Tocopherylquinone ¹³⁶	Inactive at 1000
9 Methyl perinaphthenone ⁷¹³⁴	Inactive at 1000
2 Methyl 2 phytyl 2 3-dihydro 1,4 naphthoquinone ^{135 137}	50
Naphthotocopherol ^{134 137}	500
2 5 Dimethyl benzoquinone (Phlorone) ^{138 139}	3000

TABLE VII¹⁴⁰
ESTERS AND ETHERS OF HYDROQUINONES

Compound	Effective dose in γ
Sodium 2 methyl 1 4 naphthohydroquinone diphosphate ^{141 142 143}	0 5
Sodium 2 methyl 1 4 naphthohydroquinone disulfate ^{141 143}	2
Vitamin K ₁ hydroquinone diphosphoric acid ¹⁴¹	50
Sodium 2 3-dimethyl 1 4 naphthohydroquinone disulfate ¹⁴¹	500
Potassium vitamin K ₁ hydroquinone disulfate ¹⁴¹	Inactive at 500
Diacetate of 2 methyl 1 4 naphthohydroquinone ^{144 145}	1
Dibenzoate of 2 methyl 1 4 naphthohydroquinone ^{144 145}	1
Dimesitoate of 2 methyl 1 4 naphthohydroquinone ^{146 147}	300
Monoethyl ether of 2 methyl 1 4 naphthohydroquinone ¹⁴⁸	1
Dimethyl ether of 2 methyl 1 4 naphthohydroquinone ¹⁴⁸	5
Dibenzyl ether of 2 methyl 1 4 naphthohydroquinone ¹⁴⁴	7
Vitamin K ₁ hydroquinone diacetate ¹⁴⁹	2
Vitamin K ₂ hydroquinone diacetate ¹⁴⁹	3 2

(See footnotes on opposite page)

TABLE VIII
AMINO COMPOUNDS

Compound	Effective dose in g
4 Amino-2 methyl 1 naphthol hydrochloride ^{180 181 182}	About 1
4 Amino-3 methyl 1 naphthol hydrochloride ¹⁸¹	About 1
2 Methyl 1 naphthylamine ^{182 184}	5

TABLE IX¹⁸⁵
HYDRIDES OF VITAMIN K₁ AND OF METHYL NAPHTHOQUINONE

Compound	Effective dose in g
5,8-Dihydro-vitamin K ₁ ^{186 187}	4
β -5,6,7,8-Hexahydro-vitamin K ₁ ^{186 188}	1000
2 Methyl 5,8-dihydro-1,4 naphthohydroquinone ¹⁸⁷	6
2 Methyl 5,8,9,10-tetrahydro-1,4 naphthoquinone ¹⁸⁷	8
2 Methyl 5,6,7,8-tetrahydro-1,4 naphthoquinone ¹⁸⁸	500

- ¹⁸¹ I. F. Fieser, M. Tishler and W. L. Sampson *J. Biol. Chem.* **137**, 659 (1941).
- ¹⁸² I. F. Fieser, M. Tishler and W. L. Sampson *J. Am. Chem. Soc.* **62**, 996, 1628 (1940).
- ¹⁸³ M. Tishler, I. F. Fieser and N. L. Wendler *Ibid.* **62**, 2866 (1940).
- ¹⁸⁴ I. F. Fieser, W. P. Campbell, P. M. Frey and M. D. Gates *ibid.* **61**, 2, 3, 16 (1939).
- ¹⁸⁵ J. Madinaveita *Anales soc. espan. fis. quim.* **31**, 750 (1933).
- ¹⁸⁶ I. F. Fieser, M. Tishler and W. L. Sampson *J. Biol. Chem.* **137**, 659 (1941).
- ¹⁸⁷ G. J. Martin and C. F. Lischer *Ibid.* **137**, 109 (1941).
- ¹⁸⁸ I. F. Fieser, M. Tishler and W. L. Sampson *J. Am. Chem. Soc.* **62**, 1166, 1628 (1940).
- ¹⁸⁹ I. F. Fieser, M. Tishler and N. L. Wendler *Ibid.* **62**, 2861 (1940).
- ¹⁹⁰ M. Tishler, I. F. Fieser and N. L. Wendler *Ibid.* **62**, 2866 (1940).
- ¹⁹¹ M. Tishler, I. F. Fieser and N. L. Wendler *ibid.* **62**, 198^a (1940).
- ¹⁹² S. Ausbacher and E. Fernholz *J. Biol. Chem.* **131**, 399 (1939).
- ¹⁹³ H. J. Almquist *Physiol. Rev.* **21**, 194 (1941).
- ¹⁹⁴ I. F. Fieser, M. Tishler and W. L. Sampson *J. Biol. Chem.* **137**, 6, 9 (1941).
- ¹⁹⁵ I. F. Fieser and E. M. Frey *J. Am. Chem. Soc.* **62**, 228 (1940).
- ¹⁹⁶ R. H. K. Foster, J. Lee and U. V. Solmsen *Ibid.* **62**, 453 (1940).
- ¹⁹⁷ S. Ausbacher, E. Fernholz and M. A. Dolliver *Proc. Soc. Exptl. Biol. Med.* **43**, 652 (1940).
- ¹⁹⁸ I. F. Fieser, W. P. Campbell, E. M. Frey and M. D. Gates *J. Am. Chem. Soc.* **61**, 2559, 3216 (1939).
- ¹⁹⁹ S. Ausbacher, E. Fernholz and M. A. Dolliver *Ibid.* **62**, 155 (1940).
- ²⁰⁰ M. Tishler, I. F. Fieser and N. L. Wendler *Ibid.* **62**, 2866 (1940).
- ²⁰¹ M. Tishler, I. F. Fieser and W. L. Sampson *Ibid.* **62**, 1881 (1940).
- ²⁰² M. Tishler, I. F. Fieser and N. L. Wendler *Ibid.* **62**, 198^a (1940).
- ²⁰³ S. B. Binkley, D. W. MacCorquodale, L. C. Cheney, S. A. Thayer, R. W. McKee and F. A. Dossy *ibid.* **61**, 1612 (1939).
- ²⁰⁴ E. A. Dossy, D. W. MacCorquodale, S. A. Thayer, S. B. Binkley and R. W. McKee *Science* **90**, 407 (1939).
- ²⁰⁵ H. J. Almquist and A. A. Klose *Proc. Soc. Exptl. Biol. Med.* **45**, 35 (1940).
- ²⁰⁶ D. Richtert, S. A. Thayer, R. W. McKee, S. B. Binkley and E. A. Dossy *ibid.* **44**, 601 (1940).
- ²⁰⁷ M. Tishler, I. F. Fieser and N. L. Wendler *J. Am. Chem. Soc.* **62**, 866 (1940).
- ²⁰⁸ M. Tishler, I. F. Fieser and W. L. Sampson *ibid.* **62**, 1881 (1940).
- ²⁰⁹ I. F. Fieser, M. Tishler and W. L. Sampson *J. Biol. Chem.* **137**, 659 (1941).
- ²¹⁰ I. F. Fieser, M. Tishler and W. L. Sampson *J. Am. Chem. Soc.* **62**, 996, 1628 (1940).
- ²¹¹ I. F. Fieser, M. Tishler and N. L. Wendler *Ibid.* **62**, 2861 (1940).
- ²¹² M. Tishler, I. F. Fieser and N. L. Wendler *Ibid.* **62**, 2866 (1940).

TABLE X¹⁵⁹

METHYL NAPHTHOLS METHYL TETRALONES AND RELATED COMPOUNDS

Compound	Effective dose in γ
2 Methyl 1,4 naphthohydroquinone	0.5
2 Methyl 1 naphthol ¹⁶⁰ ¹⁶¹	1
3 Methyl 1 naphthol ¹⁶⁰ ¹⁶¹	0.6
4 Methyl 1 naphthol ¹⁶⁰ ¹⁶¹	Inactive at 1000
1 Methyl 2 naphthol ¹⁶⁰ ¹⁶¹	Inactive at 1000
3 Methyl 2 naphthol ¹⁶⁰ ¹⁶¹	Inactive at 1000
1 Naphthol	1000
2 Methyl 1 tetralone ¹⁶² ¹⁶¹	0.6
3 Methyl 1 tetralone ¹⁶² ¹⁶¹	1
β Methyl naphthalene	1000

12 Determination

(a) Physical Methods

Spectroscopic Examination The vitamins K₁ and K₂ and 2 methyl 1,4 naphthoquinone can be estimated by means of their absorption spectra,¹⁶ provided the compounds are essentially free from other absorbing materials and are not present in the reduced hydroquinone form. For a description of the spectra see page 455.

(b) Chemical Methods

Colorimetric Redox Method¹⁶³ ¹⁶⁴ This method involves a catalytic hydrogenation of the vitamin K quinone to the hydroquinone stage using butanol as the solvent and phenosafranin as the indicator. An excess of 2,6 dichlorophenol indophenol is added to the reduced material in the absence of air. The decrease in color is determined and this determination is a measure of the quinone originally present.

This method is of course, not very specific since all quinones present will give the same reaction. Highly colored solutions cannot be used but, after reduction the hydroquinone can be extracted with Claisen's alkali yielding colorless solutions which can then be used in this test.

¹⁵⁹ L. F. Fieser, M. L. Hiller and W. L. Sampson, *J. Biol. Chem.* **137**, 655 (1941).¹⁶⁰ M. Tishler, L. F. Fieser and N. J. Wendler, *J. Am. Chem. Soc.* **62**, 2876 (1940).¹⁶¹ M. Tishler, L. F. Fieser and W. L. Sampson, *Ibid.* **62**, 1881 (1940).¹⁶² J. I. Pinder and J. H. Singer, *Analyst* **65**, 7 (1940).¹⁶³ J. V. Neudeck, *Proc. Am. Physiol. Soc.* **1941**, 302.¹⁶⁴ N. R. Freeman and F. A. Bacher, *J. Biol. Chem.* **137**, 745 (1941).

Reaction with Sodium Alcoholates ^{165 166} The natural vitamins K give a purple blue color reaction with sodium ethylate and sodium methylate. The color is unstable and turns into red and finally into brown. During this reaction the vitamins are degraded to 2 methyl 3 hydroxy 1 4 naphthoquinone (phthiocol) ¹⁶⁷. This color reaction is specific for those 1 4 naphthoquinone derivatives which have β unsaturated side chains in the 3 position. Carotenoids which may hinder the quantitative determination of the vitamin may be removed when the color has reached the red brown stage by extraction with a hydrocarbon solvent. The color developed from the vitamin K remains in the alcohol phase.

The sensitivity of this color reaction can be increased by testing the 2 4 dinitro phenyl hydrazones of the vitamins instead of the free vitamin ¹⁶⁸. For this purpose an alcoholic solution of the vitamin (quinone) is mixed with a solution of 2 4 dinitro phenyl hydrazine in diluted hydrochloric acid and is gently heated. A bluish green color is then developed with sodium methylate or a green color can be produced with ammonia and amyl alcohol. The colors produced are stable.

Titanous Chloride Titration ¹⁶⁹ Vitamins K as quinones can be reduced quantitatively to the corresponding hydroquinones by means of titanous chloride the end point being shown by the use of an internal oxidation reduction indicator for example potassium indigo disulfonate or phenosafranin. The determination is carried out under carbon dioxide in alcohol acetic acid solution in the presence of sodium carbonate and sodium potassium tartrate (Rochelle salt) in order to prevent the solution from becoming too acid during the titration.

Ethyl cyano-acetate Method ^{169 170} Vitamin K in alcoholic solution containing ammonia is mixed with ethyl cyano acetate followed by the addition of alkali. A yellow color develops which is measured in a photometer. The same reagent without the addition of alkali gives a violet color which however is too unstable to be useful.

(c) Biological Methods

There are two important biological methods for the determination of vitamins K. Both of these methods measure in principle the clotting power of blood.

¹⁶⁵ F. Karrer *Helv. Chim. Acta* 22: 1140 (1939).

¹⁶⁶ H. J. Almquist and A. A. Kloss *J. Am. Chem. Soc.* 61: 1611 (1939).

¹⁶⁷ I. F. Jiles and W. P. Campbell and F. M. Levy *ibid.* 61: 2206 (1939).

¹⁶⁸ A. Novelli *Sci.* 93: 358 (1911).

¹⁶⁹ J. L. Ponder and J. H. Senger *Analyst* 65: 7 (1941).

¹⁷⁰ R. C. En *J. Chem. Soc.* 1931: 160.

1 Spontaneous Blood Clotting Time Determination In the perfected form¹⁷¹ of this assay procedure day old chicks are kept on a vitamin K deficient diet, care being taken to prevent coprophagy. Within about 15 days severe avitaminosis develops. The determination of the blood clotting time is then carried out by puncturing a wing vein and placing a tube containing the blood in a thermostat which is subjected to continuous shaking. The clotting time is then defined as the time necessary to form a solid clot. While the coagulation time of the blood of normal chicks is about 4-6 minutes, the blood of avitaminotic birds may not coagulate for several hours. The determination of the vitamin K efficacy can be carried out according to this method, using either a prophylactic or a curative procedure. The latter is usually more reliable. After oral ingestion of the vitamin preparation or after injection of the material to be tested the blood clotting time is determined after six hours¹⁷¹ or in a modified procedure after 18 hours.¹⁷ Avitaminotic birds which have previously been bled may show a decrease in the clotting time upon repeated bleeding several hours later without treatment with antihemorrhagic substances¹⁷² and therefore should not be used in actual vitamin K assays.

2 Prothrombin Clotting Time Determination In this test the actual amount of prothrombin present in blood is determined^{174, 17} and therefore the test is independent of various other factors which may affect the clotting time.¹⁷⁶ In the most convenient form of this test¹⁷⁷ the ability of chicken blood to form clots in a minimum of time is determined. The blood is obtained by decapitation or from the carotid artery and is run into a tube containing sodium oxalate solution. Thromboplastin is prepared from chicken breast muscle¹⁷⁸ or from rabbit brain and then a calcium chloride solution is added. The time from the addition of the calcium chloride until a firm clot is formed is measured. Blood of normal birds clots within 25-30 seconds.

Many variations of this procedure have been suggested and in practice each laboratory uses its own modified technic. A practical important modification¹⁷⁸ is to dilute the blood plasma with an equal amount of Ringer solution and to mix this solution with thromboplastin extracts of

¹⁷¹ S. Ansbacher *J. Nutrition* 17: 303 (1937)

¹⁷² S. A. Thayer, R. W. McKee, S. B. Brinkley, D. W. MacCorquodale and I. A. Dossy *Proc. Soc. Exptl. Biol. Med.* 40: 478 (1930); 41: 194 (1939)

¹⁷³ G. Cheney *J. Lab. Clin. Med.* 24: 919 (1930)

¹⁷⁴ A. J. Quick *Am. J. Physiol.* 118: 260 (1937)

¹⁷⁵ A. J. Quick *J. Biol. Chem.* 34: LXXXVIII (1940)

¹⁷⁶ R. T. Tidrick, I. T. Joyce and H. P. Smith *Proc. Soc. Exptl. Biol. Med.* 42: 853 (1933)

¹⁷⁷ H. I. Almquist and A. A. Klotz *Biochem. J.* 33: 105 (1939)

¹⁷⁸ H. I. Almquist and A. A. Klotz *ibid.* 32: 1018 (1938)

different concentrations in a series of parallel experiments. The concentration which produces blood clotting in a defined length of time for example, within three minutes, is a measure of the amount of prothrombin present.

In the curative procedure the effect of vitamin K may be determined after one¹⁷⁹ or four days. While as pointed out above severe bleeding may reduce the whole blood clotting time no such effect has been observed on the prothrombin time.¹⁸⁰

Besides chicks, rabbits have also been recommended for use in this assay procedure.¹⁸¹ A photoelectric apparatus has also been devised for registering the clotting time.¹⁸²

The symptoms of a vitamin K deficiency can be obtained besides by dietary means by intoxication with chloroform¹⁸³ or with *p* toluene diamine.¹⁸⁴

13 Standards

No nationally or internationally accepted standard of vitamin K has been adopted so far. Both 2 methyl 1,4 naphthoquinone¹⁸⁴ ¹⁸⁵ and 2 methyl 1,4 naphthohydroquinone diacetate¹⁸⁶ ¹⁸⁷ have been proposed as a reference standard since they can be obtained easily in pure form, can be characterized by physical constants and are relatively stable.

The efficacy of various vitamin K preparations has been expressed in different units. In the table¹⁸⁸ given below these various units are correlated. The relation of these units to vitamin K₁ and to 2 methyl 1,4 naphthoquinone is given by the Thayer Doisy Unit which defines¹⁸⁹ the activity of 1 mg of vitamin K₁ as 1000 Thayer Doisy Units. The efficacy of 2 methyl 1,4 naphthoquinone is about 3.3 times as great as that of vitamin K₁.

¹⁷⁹ A. J. Quick *J. Biol. Chem.* 133: 78 (1940).

¹⁸⁰ M. C. Elliott, B. Isaacs and A. C. L. y. *Proc. Soc. Exp. Biol. Med.* 43: 40 (1940).

¹⁸¹ P. Meunier, H. Hingl and D. Bovet and A. Dreyfus *Compt. rend.* 210: 454 (1940).

¹⁸² K. K. Nygaard *J. Lab. Clin. Med.* 24: 517 (1939).

¹⁸³ H. P. Smith, E. D. Warner and K. M. Brinkhous *J. Exp. Med.* 66: 803 (1937).

¹⁸⁴ S. A. Thayer, S. B. Binkley, D. W. MacCorquodale, R. A. Doisy, A. D. Emmett, R. A. Brown and O. D. Bird *J. Am. Chem. Soc.* 61: 2563 (1939).

¹⁸⁵ E. Fernholz, A. Ausbacher and H. B. MacPhillamy *ibid.* 62: 430 (1940).

¹⁸⁶ H. Dam, J. Glavind and P. H. arr. *Helv. Chim. Acta* 23: 274 (1940).

¹⁸⁷ D. T. Ewing, J. M. Vandenberg and O. K. mm. *J. Biol. Chem.* 131: 345 (1939).

¹⁸⁸ S. Ausbacher *Proc. Am. Soc. Biol. Chem.* 1940: 111.

¹⁸⁹ S. A. Thayer, S. B. Binkley, D. W. MacCorquodale, R. A. Doisy, A. D. Emmett, R. A. Brown and O. D. Bird *J. Am. Chem. Soc.* 61: 2563 (1939).

- 1 Thayer Doisy Unit¹⁹⁰ = 0.5 Ansbacher Unit¹⁹⁶
 = 0.5 Thayer Unit (1938)¹⁹¹
 = 10 Dam Units¹⁹²
 = 0.25 Dann (1938) Unit¹⁹³
 = 0.625 Dann (1939) Unit¹⁹⁴
 = 0.08 ml. Almquist Reference Standard

14 Physiology of Plants and Microorganisms

Plants and many microorganisms produce relatively large amounts of vitamins K. This suggests that this vitamin is needed for the maintenance of life, and has actually been found to be a growth factor for *Johnes bacillus*¹⁹. No information is available which would indicate the action of this compound in plants.

15 Animal Physiology

(a) Metabolism of Vitamins K

Vitamin K occurs in the intestinal tract from the daily food intake and from synthesis by microorganisms in the bowels. Since the natural vitamins K are fat soluble compounds, they are absorbed only in the presence of bile salts^{196, 197} especially desoxy cholic acid¹⁹⁸. Thus, in rats with bile fistula vitamin K deficiency occurs even when large doses are given orally unless bile salts are administered simultaneously. Besides the presence of sufficient amounts of bile normal digestion of fats and apparently also a proper functioning of the liver¹⁹⁹ are necessary for vitamin K absorption. Intestinal lesions interfere with vitamin K absorption. The feeding of high doses of mineral oil together with vitamin K prohibits the normal absorption²⁰⁰. This effect is not noted when the vitamin is injected. Vitamins K are generally active upon parenteral injection. Water soluble forms of vitamin K are absorbed from the intestinal tract

¹⁹⁰ S. Ansbacher *Proc. Soc. Exptl. Biol. Med.* **44**, 48 (1940)

¹⁹¹ S. A. Thayer, D. W. McCorquodale, R. W. McKee and H. A. Doisy *J. Biol. Chem.* **123**, CXX (1938)

¹⁹² H. Dam and J. Glavand *Z. Vitaminforsch.* **9**, 71 (1933)

¹⁹³ F. P. Dann *Am. J. Physiol.* **123**, 48 (1938)

¹⁹⁴ F. P. Dann *Proc. Soc. Exptl. Biol. Med.* **42**, 613 (1934)

¹⁹⁵ D. W. Woolley and J. R. McCarter *Ibid.* **45**, 357 (1940)

¹⁹⁶ J. D. Greaves and C. L. A. Schmidt *Ibid.* **37**, 43 (1937)

¹⁹⁷ H. R. Butt *Am. J. Digestive Diseases Nutrition* **6**, 127 (1939). H. R. Butt, A. M. Snell and A. E. Osterberg *Proc. Staff Meetings Mayo Clinic* **13**, 74 (1938)

¹⁹⁸ C. L. A. Schmidt *Pacific Coast Med.* **5**, 7 (1935)

¹⁹⁹ R. L. Clark, C. F. Dixon, H. R. Butt and A. M. Snell *Proc. Staff Meetings Mayo Clinic* **14**, 407 (1939)

²⁰⁰ M. C. Elliott, H. Isaac and A. C. Ivy *Proc. Soc. Exptl. Biol. Med.* **43**, 240 (1940)

even in the absence of bile salts²⁰¹ ²⁰² and are especially useful for intra venous injections

Vitamins K are found only in very small amounts in blood (apparently only during times of transport) but in somewhat larger amounts in livers. The animal organism has apparently no storage organs for this vitamin and metabolizes this compound fairly rapidly. The mechanism of this metabolism is unknown. No vitamin K is excreted through the kidneys²⁰³, but considerable amounts are found in feces mainly due to the bacterial synthesis in the intestinal tract.

Vitamin K is secreted in small but definite amounts in milk and eggs²⁰⁴. There is apparently a special mechanism which regulates the passage of this vitamin through the placenta. It has generally been observed that pregnant women just prior to parturition have an increased need for vitamin K. The newborn infant usually has a slight K hypovitaminosis which can be overcome by feeding vitamin K to the infant or by giving it to the mother before delivery. Better results are usually obtained by maternal administration than by giving vitamin K after birth²⁰⁵.

(b) *Physiological Action of Vitamins K*

Vitamins K are necessary for the maintenance of normal blood coagulation. According to the classical theory²⁰⁶ two different phases are involved in this process of coagulation. The end effect which constitutes the second phase of the total process is the transformation of fibrinogen, a protein dissolved in the blood plasma, into fibrin, a solid and essentially insoluble protein derivative. This precipitation reaction is carried out by a postulated enzyme, the thrombin. All experimental evidences point to the actual existence of this compound. Thrombin does not occur in the blood, since otherwise the blood would not be liquid. But the precursor of thrombin, namely prothrombin is present in plasma. The first phase of the blood coagulation therefore, is the conversion of prothrombin into thrombin. This process involves the action of another enzyme, thromboplastin (or thrombokinase) in the presence of calcium ions. While the latter are present in blood plasma, thromboplastin is a normal cell constituent but not a blood constituent.

²⁰¹ E. D. Warner, K. M. Brinkhous and H. P. Smith *Proc. Soc. Exptl. Biol. Med.* 44: 607 (1941).

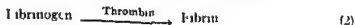
²⁰² H. P. Smith and C. A. Owen *J. Biol. Chem.* 134: 783 (1940).

²⁰³ H. Dam, A. Geiger, J. Glavud, P. Karrer, W. Karrer, E. Rothschild and H. Salomon *Helv. Chim. Acta* 22: 310 (1939).

²⁰⁴ H. J. Almquist and E. L. R. Stoksted *J. Nutrition* 12: 379 (1936).

²⁰⁵ L. B. Shettles, E. Delfs and L. M. Hellman *B. H. J. Am. Hosp. & Hosp.* 65: 419 (1939).

²⁰⁶ O. Hammenstein *Z. phys. Chem.* 28: 98 (1899). P. Morawitz *Biochem. Z.* 18: 30 (1909).



During vitamin K deficiency only the prothrombin concentration is altered, that is, reduced²⁰⁷ Vitamin K however is not identical with prothrombin since this vitamin does not cause blood coagulation *in vitro*²⁰⁸ Vitamin K,²⁰⁷ apparently, is also not a constituent of prothrombin since prothrombin fractions exert practically no vitamin K action²⁰⁹ Since however, vitamin K influences the prothrombin concentration it is assumed that vitamin K is involved in the prothrombin formation This synthesis is apparently accomplished in the liver,²⁰⁹ ²¹⁰ since partial extirpation of the liver markedly reduces the prothrombin level in blood²¹¹ ²¹² and since symptoms of a vitamin K deficiency even in the presence of normally adequate amounts of this vitamin have been observed frequently in cases of liver damages such as cirrhosis, hepatitis, atrophy and carcinoma²¹³ In all these cases the prothrombin concentration could not be influenced by vitamin K when given orally or injected intravenously or intramuscularly That a special mechanism for the formation of prothrombin actually exists is furthermore suggested by the fact that excessive feeding of vitamins K will not cause the prothrombin to rise above a certain level²¹⁴

The mechanism of the vitamin K action is unknown It has been suggested¹³ that the quinone structure of the vitamin takes part in an oxidation reduction system involving the oxidation of SH groups to —S—S linkages The hypothesis has been advanced²¹⁵ that the formation of the blood clot also involves an oxidation of SH groups of fibrinogen to —S—S groups in fibrin

²⁰⁷ H Dam *Nature* 135 652 (1935) F Schönheyder *Ibid* 135 652 (1935) H Dam F Schönheyder and E Tage Hansen *Biochem J* 30 1075 (1936) F Schönheyder *Ibid* 30 890 (1936)

²⁰⁸ H Dam J Clavind L Lewis and E Tage Hansen *Skand Arch Physiol* 79 121 (1938)

²⁰⁹ C L A Schmidt *Pacific Coast Med* 5 7 (1938)

²¹⁰ A M Snell *J Am Med Assoc* 112 1457 (1939)

²¹¹ W D Andrus J W Lord and R A Moore *Surgery* 6 899 (1939)

²¹² E D Warner *J Exptl Med* 68 831 (1938)

²¹³ P M Aggeler S P Lucia and L Goldman *Proc Soc Exptl Biol Med* 43 689 (1940) W D Andrus and J W Lord *J Am Med Assoc* 114 1336 (1940) H R Butt A M Snell A E Osterberg and J L Bollman *Proc Staff Meetings Mayo Clinic* 15 69 (1940) F J Pohle and J K Stewart *J Clin Investigation* 19 36 (1940) G H Scanlon E M Brinkhous W D Warner H P Smith and J E Flynn *J Am Med Assoc* 112 1898 (1939)

²¹⁴ A J Quick *Proc Am Soc Biol Chem* 34 LXXXVIII (1940)

²¹⁵ F Bernheim and M L C Bernheim *J Biol Chem* 134 4 7 (1940)

²¹⁶ J P Baumberger *Proc Am Physiol Soc* 1941, 18

16 Hypovitaminosis and Avitaminosis

In vitamin K deficiency the prothrombin content of blood is markedly decreased and the blood clotting time is considerably prolonged. The principle symptom during times of vitamin K deficiency is, besides a prolonged bleeding time, the occurrence of hemorrhages. In chicks subcutaneous and intramuscular hemorrhages occur on the head, neck, breast, abdomen, back, wings and legs.¹⁷ In man hemorrhagic syndromes caused by a vitamin K deficiency are frequently observed in an infant during the first few days of life when an extremely low prothrombin concentration generally is observed which lasts until a normal intestinal flora has been developed by the ingestion of food.^{18 219 220 221} but they may also occur in adults.² Low prothrombin levels are furthermore generally observed in humans when the absorption of the vitamin is impaired, such as in cases of obstructive jaundice,^{22 224 225} sprue,²²⁶ biliary fistula,^{227 228} and ulcerative colitis. Hemophilia and thrombopenia however are not due to vitamin K deficiency and cannot be influenced by the administration of this vitamin.^{229 30 31}

(a) Clinical Test Methods

1. **Bleeding Time Determination** A stab is made in the tip of a finger and the amount of time which it takes to stop the bleeding is observed. In order to obtain measurable figures it has been suggested that the blood be absorbed every 15 seconds on a piece of filter paper. The

¹⁷ S. Ausbacher *J. Nutrition* 17: 303 (1937)

¹⁸ C. D. Johnson *J. South Carol. Med. Assoc.* 36: 336 (1940)

¹⁹ H. Dam *et al.* *Lancet* 2: 1157 (1939)

²⁰ W. W. Waddell and D. Guerry *J. Pediat.* 15: 80 (1939)

²¹ A. J. Quick and A. M. Grossman *Am. J. Med. Sci.* 199: 1 (1940) *Proc. Soc. Exptl. Biol. Med.* 40: 647 (1939) 41: 227 (1939)

²² R. Kark and E. L. Lozner *Lancet* 2: 1162 (1939)

²³ S. T. Townsend and E. S. Mills *Can. Med. Assoc. J.* 41: 111 (1939) 42: 541 (1940)

²⁴ K. M. Brinkhous, H. P. Smith, and E. D. Warner *Am. J. Med. Sci.* 196: 50 (1938) E. D. Warner, K. M. Brinkhous, and H. P. Smith *Proc. Soc. Exptl. Biol. Med.* 37: 828 (1938) A. M. Soell, T. B. Magath, E. W. Boland, A. E. Osterberg, H. R. Butt, J. L. Bollman, and W. Walters *Proc. Staff Meet. at Mayo Clinic* 13: 65 (1938)

²⁵ A. J. Quick, M. Stanley Brown, and F. W. Bancroft *Am. J. Med. Sci.* 190: 501 (1935)

²⁶ P. M. Aggeler, S. P. Lucia, and L. Goldman *Proc. Soc. Exptl. Biol. Med.* 43: 689 (1940) R. L. Clark, C. F. Dixon, H. R. Butt, and A. M. Soell *Proc. Staff Meetings Mayo Clin.* 14: 407 (1939) R. Engel *Med. Welt* 13: 120 (1939) H. Hult *Acta Med.* 3: 2428 (1939)

²⁷ I. C. Zuckerman, B. Kogut, M. Jacobson, and J. Y. Cohen *Am. J. Digest. Dis.* 6: 337 (1933)

²⁸ L. K. Ferguson and D. G. Calder *ibid.* 6: 722 (1939) H. P. Smith, S. R. Ziffer, C. A. Owen, G. R. Hoffman, and J. E. Flynn *J. Iowa State Med. Soc.* 29: 377 (1939)

²⁹ H. Dam and J. Glavind *Ugeskrift Læger* 100: 248 (1938)

³⁰ H. Dam, P. Schaubeyder, and E. Tage Jensen *Biochem. J.* 30: 1075 (1936)

³¹ G. H. Scanlon, K. M. Brinkhous, E. D. Warner, H. P. Smith, and J. E. Flynn *J. Am. Med. Assoc.* 112: 1898 (1939)

blood drops should become continually smaller. The bleeding normally stops after 1-3 minutes. Continued bleeding may be due to many causes such as deficiency in vitamins C or K, hemophilia, etc.

2 Coagulation Time Determination About 1 cc of fresh blood is drawn in a tube containing thromboplastin. The tube is shaken and the clotting time is observed. The determination is carried out with the blood of a normal person as a control. This test²³² has been recommended²³³ as a bedside method since it is easy to carry out and is very reliable.

3 Prothrombin Time Determination This method has already been described among the methods used for the determination of vitamin K and has been most successfully used clinically in various modifications.²³³⁻²³⁶ Various micromethods have been worked out²³⁷ which require only one drop of blood.

4 Two-Stage Determination of Prothrombin In this test²³⁸ the blood is first mixed with a sodium oxalate solution and is centrifuged. The plasma is then defibrinated by adding thrombin. The remaining fluid contains the prothrombin which is completely converted into thrombin by mixing various dilutions with exactly defined solutions of saline, calcium ions and thromboplastin. A specified amount of fibrinogen is then added and the amount of thrombin previously formed is measured by determination of the clotting time.

5 Serum Volume Test²³⁹ The volume of the blood serum adjusted to the red cell count, decreases during early stages of vitamin K hypovitaminosis and its determination in conjunction with an examination of the blood clot for friability has been claimed to serve as an indication of danger of bleeding.

17 Hypervitaminosis

The naturally occurring vitamins K₁ and K₂ are non toxic, even when given in excessive doses.⁴⁰ Thus in mice no lethal effect could be pro-

²³² H. P. Smith, S. L. Ziffern, C. A. Owen and G. R. Hoffman, *J. Am. Med. Assoc.* 113: 383 (1933).
S. E. Ziffern, C. A. Owen, G. R. Hoffman and H. P. Smith, *Proc. Soc. Exptl. Biol. Med.* 40: 591 (1939).

²³³ H. R. Butt and A. M. Snell, *Vitamin K*, Philad. Iphia, 1941.

²³⁴ A. J. Quick, M. Stanley Brown and F. W. Bancroft, *Am. J. Med. Sci.* 190: 501 (1935).

²³⁵ A. J. Quick, *Am. J. Clin. Path.* 10: 222 (1940).

²³⁶ T. B. Magath, *Am. J. Clin. Path.* (Tech. Suppl.) 3: 187 (1939).

²³⁷ A. J. Quick, *Proc. Soc. Exptl. Biol. Med.* 42: 788 (1939). W. E. Bray and O. R. Kelly, *Am. J. Clin. Path.* 10: 154 (1940). O. R. Kelly and W. E. Bray, *J. Lab. Clin. Med.* 25: 27 (1940). K. Kato, *Am. J. Clin. Path.* 10: 147 (1940).

²³⁸ E. D. Warner, K. M. Brinkhous and H. P. Smith, *Am. J. Physiol.* 114: 667 (1936).

²³⁹ E. F. Boyce and E. M. McFetridge, *New Orleans Med. Surg. J.* 91: 177 (1939).

⁴⁰ S. Ansbacher, *Proc. Am. Soc. Biol. Chem.* 1940: 111.

duced with doses as high as 20 g of vitamin K₁ per kilogram of body weight²⁴¹ (studies on man⁴) The easily available substitutes 2 methyl 1 4 naphthoquinone and the 2 methyl 1 4 naphthohydroquinone however show definite toxic symptoms^{43 241} when fed to dogs rabbits or humans in excessive amounts Vomiting and porphyrinuria and occasionally albuminuria have been observed when 180 mg of these compounds was given orally to humans or when 30-60 mg per kilogram body weight was fed to dogs In rabbits excess feeding of 2 methyl 1 4 naphthoquinone has been noted to cause a prolongation of the blood clotting time which amounts to an effect which is contrary to that obtained by feeding normal doses of vitamin K²⁴⁵ In mice small quantities of 2 methyl 1 4 naphthoquinone and similarly also phthiocol cause a drop in the erythrocyte count and hemoglobin while quantities from 0 2 to 0 5 g per kilogram body weight cause death²⁴¹

18 Requirements

Vitamins K are required by all animals experimentally investigated such as the chick duck goose, canary, pigeon,⁴⁶ turkey,⁴⁷ rat^{248 249} rabbit,⁵⁰ mouse²⁴¹ dog⁵ and man^{2 3} The actual amount of this vitamin required by different species is unknown In human therapy amounts varying from 1 to 10 mg of 2 methyl 1 4 naphthoquinone have been recommended²⁴ In general pregnant and lactating women need increased amounts of this vitamin in order to protect the newborn from hypovitaminosis (See also page 614)

Vitamins K are not required by most microorganisms such as yeast fungi and most bacteria but seem necessary for growth of *Johnes bacillus*⁵¹

²⁴¹ H Molitor and H J Robinson *Proc Soc Exptl Biol Med* 43 1 (1940)

⁴ H R Butt and A M Smith *Vitamin K* Phad lph 1941

⁴³ Koller Schweiz med Wochsch 45 11 (1913)

²⁴⁵ H Molitor and H J Robinson *Proc Soc Exptl Biol Med* 43 1 (1940)

²⁴ Meiner H Hinglas D Bovet and A Dreyfus *Compt Rend* 210 454 (1940)

⁴⁶ H Dam F Schönbeyr and J Lewis *Arch m J* 31 72 (1937)

⁴⁷ J Almquist *Proc Swedish Acad Sci Poultry Cress Cl* 1939 138

⁵⁰ H Dam and J Glavin *Z Vitaminforsch* 9 71 (1939)

²⁴⁸ J D Caves *Am J Physiol* 125 4 2 (1939)

²⁴⁹ H D Marsd J Glavin *Acta Med Scand* 96 108 (1938)

⁵¹ R Murphy *Science* 89 203 (1939)

²⁴² W B Hawkin and K M Brinkhous *J Exptl Med* 63 795 (1936)

⁴⁸ R Ark and F L Jozner *Lancet* 237 1167 (1939)

⁴⁹ H Dam *Arch Res Biochem* 9 373 (1940)

⁵⁰ D W Woolley and J R McCart *Proc Soc Exptl Biol Med* 45 757 (1940)

VITAMIN P

VITAMIN P

1 Nomenclature and Survey

Names

Vitamin P^{1, 2}

Citrin^{1, 2}

Permeability vitamin

Composition

Vitamin P is the name given to a crude extract which contains besides other physiologically active compounds the glucosides eriodictin and hesperidin which yield upon hydrolysis eriodictyol and hesperitin

2 Chronology

- 1936 ARMENTANÓ BENTSÁTH BÉRES RUSZNYÁK and SZENT GYÖRGY^{1, 2} reported the occurrence of a substance other than vitamin C which controls hemorrhages in man. The active material was identified as a flavanone
- 1939 SCARBOROUGH³ presented evidence from experiments on human subjects which established the existence of a factor decreasing capillary fragility

3 Occurrence

The distribution of vitamin P in nature has not been investigated for a large number of foods. It has been shown^{4, 5} that this vitamin is present in citrus fruits, such as lemon, orange and grapefruit. The skin of these fruits is generally richer than the juice from the pulp. Lemon juice contains more vitamin P than does orange juice and this in turn contains more than grapefruit juice. Vitamin P is also found in the juice of other plants such as paprika and is believed to be widely distributed over the entire plant kingdom.

It has not been possible to demonstrate the presence of vitamin P (eriodictyol or hesperitin) in animal materials such as milk, liver or kidney.^{6, 7}

¹ L. Armentanó, A. Bentsáth, T. Béres and I. Rusznayák, *Deut. med. Wochschr.*, 62, 1326 (1936)

² A. Bentsáth, I. Rusznayák and A. Szent Györgyi, *Natur*, 138, 798 (1936)

³ H. Scarborough, *Biochem. J.*, 33, 1400 (1939)

⁴ I. Rusznayák and A. Szent Györgyi, *Nature*, 138, 27 (1936)

⁵ A. Szent Györgyi, *Z. physiol. Chem.*, 255, 176 (1938)

⁶ I. Robeznieks, *Z. Vitaminforsch.*, 8, 27 (1938-39)

⁷ W. Neuwessler, *Ibid.*, 9, 338 (1939)

Vitamin P appears to occur in plants predominantly in the form of glucosides⁸

4 Isolation

The isolation of vitamin P is based on its property of being precipitated by lead salts in neutral solution and by alkali in anhydrous alcoholic solution. The actual isolation procedure, for example, from lemons, is carried out as follows.⁹ The ripe fruit is pressed and the peels are ground and extracted with 96% alcohol. By the addition of Ba and Pb acetate solutions to the combined juice and extracts considerable amounts of impurities are precipitated. The vitamin is then precipitated by neutralizing the solution with ammonium hydroxide. The precipitate can be purified by reprecipitation and is then suspended in alcohol to which dilute sulfuric acid is added. Lead sulfate precipitates, and the vitamin goes into solution. The vitamin is obtained from the alcoholic solution by fractional precipitation with alkali. The free vitamin is obtained from the alkali salt by acidification. The procedure is essentially the same for the isolation of vitamin P from dried material but the operations are reversed. After an initial alcohol extraction the vitamin is precipitated with alkali, the precipitate is dissolved in acetic acid and the vitamin is precipitated with lead acetate and ammonium hydroxide.

Two final products are obtained: a crystalline material consisting essentially of hesperidin with some eriodictin,¹⁰ and the mother liquor which contains mainly eriodictin plus some hesperidin. The crystalline material and the solution apparently contain other physiologically active components in addition to eriodictin and hesperidin. An efficient method for the preparation of absolutely pure eriodictin or hesperidin has not been worked out and a separation has not been accomplished satisfactorily. The crystalline material can be recrystallized from pyridine and water.¹¹

The glucosides eriodictin and hesperidin yield upon hydrolysis with dilute acids eriodictyol and hesperitin.

5 Properties

The chemical and physical properties of vitamin P cannot be stated since the chemistry is not definitely established. Concentrates contain eriodictin and hesperidin besides other unknown components.

⁸ V. Bruckner and A. Szent-Györgyi, *Nature* 138, 1057 (1936).

⁹ A. Szent-Györgyi, *Z. physiol. Chem.* 255, 126 (1938).

¹⁰ V. Bruckner and A. Szent-Györgyi, *Nature* 138, 1057 (1936).

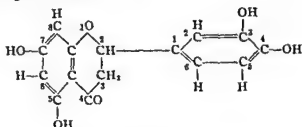
¹¹ H. Scarborough, *Biochem. J.* 33, 1400 (1939).

Eriodictyol forms light yellow crystals which melt at 267° C. These crystals are sparingly soluble in water, but very soluble in organic solvents and in alkali.

Hesperitin melts at 224° C. and is readily soluble in water and in organic solvents.

6 Constitution

There are probably two glucosides which are vitamin P^{11a} namely *hesperidin* which is hesperitin 1 rhamnoside glucose, and *eriodictin* which is eriodictyol 1 rhamnoside. Indications are that these compounds may be active only when the flavanone rings are opened to the corresponding chalcones. In the glucosides the sugar residues are attached to the 7 position. Hesperitin is the 4' methyl ether of eriodictyol. The latter has the following constitution:



Eriodictyol
5734 Tetrahydroxy flavanone

7 Biogenesis

The method which is used in the plant organism for the synthesis of vitamin P is not known. Various theories concerning the formation of flavanones in plants have been advanced.¹² The biogenetic scheme implies a synthesis from carbohydrates. Specifically the flavanones appear to be built up from two hexoses and one triose by means of aldol condensations. Coniferyl alcohol, a compound which is believed to be a building unit of lignin, may be an intermediate.

8 Specificity

Little is known about the specificity of vitamin P. Besides the hydrolyzed vitamin concentrate containing eriodictyol and hesperitin, the naturally occurring glucosides have been shown to be active.

^{11a} A. M. G. *Z. Phys. Chem.* **274**, 109 (1942); C. Zemplén and R. Fogar, *Ber.* **76**, 773 (1943); C. Z. Wawer and J. I. Webb, *Sci.* **96**, 307 (1941); R. H. H. Ghy, *J. Am. Pharm. Assoc.* **32**, 74 (1942).

¹² R. Robinson, *Nature* **137**, 172 (1936).

9 Determination

(a) Chemical Methods

There is no chemical method by which vitamin P can be detected. This is due mainly to the fact that the chemical composition of vitamin P is not known. Certain color reactions for the flavanones, such as the ones isolated from vitamin P preparations, cannot be accepted as tests for the vitamin as long as their vitamin nature is not better defined.

Vitamin P is said to give a yellow color with boric acid¹³. This color reaction may be used for the determination of flavones in natural products. The flavanones, on the other hand, give no color reaction with boric acid. Since vitamin P preparations contain predominantly flavanones (eriodictin and hesperidin), the positive color reaction must be due to some other unknown constituent.

(b) Biological Methods

The biological methods recommended for the demonstration of the presence of vitamin P are based on the fact that during vitamin P deficiency the capillary resistance of experimental animals is diminished. The occurrence of hemorrhages (type and number) has also been used as a criterion for vitamin P deficiency but with less success^{14, 15, 16}. Guinea pigs have been used predominantly^{17, 18, 19, 20} as experimental animals but the rat responds equally well to a vitamin P deficient diet and can be healed by the administration of this vitamin.

10 Standards

No standard of vitamin P has been designated.

11 Physiology of Plants

The physiological reason for the presence of vitamin P in the plant organism is not known. The flavanones may constitute structural building units.

¹³ C. W. Wilson *J. Am. Chem. Soc.* 61: 2303 (1939).

¹⁴ A. Szent-Györgyi *Z. physiol. Chem.* 255: 126 (1938).

¹⁵ S. S. Zilva *Biochem. J.* 31: 915 (1937).

¹⁶ T. Moll *Klin. Wochschr.* 16: 1653 (1937).

¹⁷ A. Bentsáth, I. Rusznayák and A. Szent-Györgyi *Nature* 138: 798 (1936).

¹⁸ A. Bentsáth, I. Rusznayák and A. Szent-Györgyi *Ibid.* 139: 326 (1937).

¹⁹ E. N. Todhunter, R. C. Robbins, G. Ivey and W. Brewer *J. Nutrition* 19: 113 (1940).

²⁰ A. Bentsáth and N. B. Das *Z. physiol. Chem.* 247: 258 (1937).

²¹ C. E. Zacho *Acta Path. Microbiol. Scand.* 16: 1411 (1939).

²² I. Rusznayák and A. Benko *Science* 94: 26 (1941).

It has been suggested²³ that vitamin P acts in plants as a detoxifying agent and that considerable amounts of this agent are held available for times of need in the form of a glucoside. This is feasible since certain other biologically important compounds are stored in the plant organism in the form of inactive glucosides and, when needed, the glucosides are hydrolyzed and the biologically active compounds are set free.

12 Animal Physiology

Vitamin P (and probably the glucosides after hydrolysis in the intestines) is absorbed from the intestinal tract. Excess amounts are excreted through the urine.⁴⁻⁵ Vitamin P is also active when given by intramuscular or intravenous injection or through the rectum. The physiological action of vitamin P is concerned with the maintenance of normal conditions in the walls of the small blood vessels and the absence of this vitamin causes increased capillary fragility and permeability. It has been suggested that the action of vitamin P in protecting capillary resistance may be due to a detoxifying power.²⁶ The action of vitamin P, however, is not due to a change in concentration of any of the blood clotting factors such as prothrombin, fibrinogen or platelets. It has been demonstrated on the other hand, that vitamin P is able to increase the calcium level in the blood.²⁷ The view has also been expressed that vitamin P may be required for the absorption and retention of ascorbic acid.³

Vitamin P but apparently not the isolated constituents hesperidin and eriodictin causes upon intravenous injection a definite drop in blood pressure⁹⁻²⁰ as demonstrated on the cat, rabbit, frog and turtle.²¹

Vitamin P also promotes growth and cell division in the fertilized ovum of sea urchins.³²

13 Avitaminosis

A state of vitamin P deficiency is known for the guinea pig, rat and for the human organism. In patients suffering from vitamin P deficiency

¹ I. N. Kugelmass *J. Am. Med. Assoc.* 115: 513 (1940).

² I. Armentano, E. B. Hatz and I. Rusznyak *Alim. Hochsch.* 17: 733 (1938).

³ I. Huzsak *Z. physiol. Chem.* 249: 14 (1937).

⁴ I. N. Kugelmass *J. Am. Med. Assoc.* 115: 519 (1940).

⁵ M. Raunert *Z. Urol.* 32: 630 (1938).

⁶ A. Elmby and E. Warburg *La cel.* 1937: 11: 1363.

⁷ A. Sz. ut-Györgyi *Z. physiol. Chem.* 255: 16 (1938).

⁸ I. Armentano *Z. ges. u. pfl. Med.* 102: 219 (1937).

⁹ A. J. Leser, C. F. Lombard, C. H. Thien and J. L. Webb *Proc. Am. Soc. Pharmacol. Exptl. Therap.* 1941: 26.

¹⁰ F. Ludwig *Arch. u. pfl. Path. Pharm. kol.* 129: 113 (1938).

capillary resistance is decreased and vascular permeability is increased. The clinical symptoms comprise hemorrhagic conditions of the skin (nutritional purpura) which cannot be cured by vitamin C.^{33 34 35 36 37 38} Vitamin P therapy has also been found of value in hemorrhagic conditions of otherwise non specific character, such as in cases of bleeding in the kidney (nephritis) and in the stomach.³⁹

14 Requirements

The exact vitamin P requirement of the human organism is not known. In clinical cases 50–150 mg of the natural mixture of compounds have been administered. Rats with diminished capillary resistance have been given 3–4 mg per day subcutaneously.

³³ L. Armentanó, A. Bentsáth, T. Héres and I. Rusznyak *Deut med Wochschr* 62 1326 (1936)

³⁴ H. Scarborough *Biochem J* 33 1400 (1939)

³⁵ I. N. Kugelmass *J Am Med Assoc* 115 519 (1940)

³⁶ S. Lajos *Klin Wochschr* 16 1615 (1937)

³⁷ C. T. Decker *Munch med Wochschr* 86 292 (1939)

³⁸ T. Jersild *Lancet* 1938 I 1445

³⁹ M. Raunert *Z Urol* 32 630 (1938)

**NON-IDENTIFIED
VITAMINS**

THE NON-IDENTIFIED VITAMINS

In addition to the vitamins which are identified and which have been discussed in the preceding chapters of this monograph, there is an unknown number of non identified vitamins. Their existence has as a general rule been postulated since it was found that an animal species kept on a more or less purified diet ceased to grow or developed specific diseases which could be alleviated by the feeding of special foods. As a result a great number of unknown vitamins have been claimed. It remains to be seen how many of these dietary essentials will prove to be separate entities. On the other hand there is the definite possibility that future research will uncover the existence of other vitamins the existence of which is unknown today.

1 Vitamin B₁

In the early work on vitamin B₁ from 1911 to 1920 it was noted¹ that an additional factor (called vitamin B₂²) was necessary in order to cause pigeons to gain weight. Vitamin B₁ was described by Williams and Waterman in 1928.³ A clearer definition of this vitamin was offered in 1936 by Carter and O'Brien.⁴ Vitamin B₁ occurs in liver, yeast, whole grains and malt. It is soluble in water and dilute alcohol and sensitive to heat and alkali. Vitamin B₁ is present in the filtrate fraction after adsorption of an extract on fuller's earth.⁵ It is possible that vitamin B₁ is identical with pantothenic acid.

2 Vitamin B₂

The existence of a special factor (or factors) designated as vitamin B₂ which prevents the occurrence of a typical paralysis in rats⁶ and in chicks⁷

¹ H. Schaumann *Trans. Soc. Tropical Med. Hyg.* 5: 59 (1911). F. A. Cooper *J. Hyg.* 12: 436 (1912). A. D. Emmett and L. H. McKim *J. Biol. Chem.* 32: 409 (1917).

² R. R. Williams and W. H. Eddy *Connecticut Inst. Wash. Yearbook* 27: 378 (1928).

³ R. R. Williams and R. F. Waterman *J. Biol. Chem.* 78: 311 (1928).

⁴ C. W. Carter and J. R. O'Brien *Biochem. J.* 30: 43 (1936).

⁵ C. W. Carter and J. R. O'Brien *Ibid.* 33: 1810 (1939).

⁶ V. Reader *Biochem. J.* 23: 689 (1929). O. L. Kline, C. A. Elvehjem and F. B. Hart *Ibid.* 30: 780 (1936).

⁷ J. A. Keenan, O. L. Kline, C. A. Elvehjem and F. B. Hart *J. Biol. Chem.* 103: 671 (1933).



THE NON-IDENTIFIED VITAMINS

In addition to the vitamins which are identified and which have been discussed in the preceding chapters of this monograph there is an unknown number of non identified vitamins. Their existence has, as a general rule, been postulated since it was found that an animal species kept on a more or less purified diet ceased to grow or developed specific diseases which could be alleviated by the feeding of special foods. As a result, a great number of unknown vitamins have been claimed. It remains to be seen whether many of these dietary essentials will prove to be separate entities. On the other hand there is the definite possibility that future research will discover the existence of other vitamins the existence of which is unknown.

1 Vitamin B₁

In the early work on vitamin B₁ from 1911 to 1920 it was noted¹ that an additional factor (called vitamin B₂²) was necessary in order to cause rats to gain weight. Vitamin B₂ was described by Williams and Sherman in 1928.³ A clearer definition of this vitamin was offered in 1931 by Carter and O'Brien.⁴ Vitamin B₂ occurs in liver, yeast, whole wheat and malt. It is soluble in water and dilute alcohol and sensitive to heat and alkali. Vitamin B₂ is present in the filtrate fraction after addition of an extract on fuller's earth.⁵ It is possible that vitamin B₂ is identical with pantothenic acid.

2 Vitamin B₄

The existence of a special factor (or factors) designated as vitamin B₄ which prevents the occurrence of a typical paralysis in rats⁶ and in chicks⁷

¹ Schaumann *Trans Soc Trop Med Hyg* 5:59 (1911). ² A. Cooper *J Hyg* 12:436

³ A. D. Emmett and L. H. McKim *J Biol Chem* 32:409 (1917).

⁴ R. Williams and W. H. Eddy *Carnegie Inst Wash Yearbook* 27:378 (1928).

⁵ R. Williams and R. F. Waterman *J Biol Chem* 78:311 (1928).

⁶ W. Carter and J. R. O'Brien *Biochem J* 30:43 (1936).

⁷ W. Carter and J. R. O'Brien *Ibid* 33:1810 (1939).

⁸ Reader *Biochem J* 23:689 (1929). O. L. Kline, C. A. Elvehjem and E. B. Hart *Ibid* 30:780

⁹ A. K. Tuan, O. L. Kline, C. A. Elvehjem and E. B. Hart *J Biol Chem* 103:671 (1933).

has been assigned to this compound. Adenine has been shown to be necessary for certain strains of lactic acid bacteria¹⁷ and it has been claimed that adenylic acid is the coenzyme of the fatty acid dehydrogenase.¹⁸ In clinical studies¹⁹ on pellagrins adenylic acid seemed to increase the effect of nicotinic acid. When 3 to 20 mg of yeast adenylic acid were injected intravenously into patients deep involuntary, gasping inspiration a fluttering sensation in the upper part of the abdomen and a feeling of fullness in the head were observed.

6 Vitamin B₆

The occurrence of anemia has been observed in chicks as the result of a deficiency of an unknown member of the vitamin B complex.²⁰ The postulated vitamin is present in liver but has not been identified. The deficiency syndrome comprises a decreased red blood cell count, decreased per cent of hemoglobin in the blood and decreased red cell volume. In addition to showing the symptoms of anemia young animals deficient in this vitamin grow only slowly.

7 Vitamin B₇ (Anti-Perosis Vitamin)

Perosis, a deficiency disease characterized by the occurrence of deformed legs and known also as hock disease or slipped tendon, has frequently been observed in chicks on synthetic diets.²¹ The lower bones in perotic chicks are abnormally short and twisted. Perosis can be treated to a certain extent prophylactically by manganese² and by choline.²² In addition there seems to exist another perosis preventing compound of organic chemical nature.²⁴

8 Vitamin J²⁵

Vitamin J, which has at times also been called vitamin C₂, has been postulated as an anti pneumonia factor. The existence of such a factor has been

¹ E. F. Möller *Z. angew. Chem.* 52, 466 (1939).

¹⁸ K. Lang and H. Mayer *Z. physiol. Chem.* 262, 120 (1939).

¹⁹ T. D. Spies, D. P. Hightower and L. H. Hubbard *J. Am. Med. Assoc.* 115, 292 (1940).

²⁰ A. G. Hogan and E. M. Parrott *J. Biol. Chem.* 132, 507 (1940).

²¹ A. G. Hogan, N. B. Guerrant and H. L. Kempster *Ibid.* 64, 113 (1945). A. G. Hogan and C. L. Shrewsbury *J. Nutrition* 3, 39 (1930). A. G. Hogan, L. R. Richardson and H. Patrick *Ibid.* 19, Proc. 14 (1940).

²² H. S. Wilgus, I. C. Norris and G. I. Heuser *J. Nutrition* 14, 10 (1937).

²³ T. H. Jukes *J. Biol. Chem.* 134, 783 (1940).

²⁴ A. G. Hogan, L. R. Richardson, H. Patrick and H. L. Kempster *J. Nutrition* 21, 327 (1941).

²⁵ H. v. Euler, H. Soder and M. M. Imberg *Z. Hyg. Infekt. Krankh.* 116, 672 (1935).

has been postulated. This factor is present in yeast and in liver. Dried grass, wheat germ, pork brain and pork kidney are good sources while grains are relatively poor sources.⁸ Rats on a vitamin B₄ deficient diet show general muscular weakness, spastic gait, swollen paws and a tendency to sit in a hunched position.⁹ Chicks which are deficient in vitamin B₄ show a disturbed gait, a lack of coordination and a tendency to fall on their side with their legs in tension pulled against the abdomen. It has been suggested¹⁰ that vitamin B₄ may be identical with a mixture of arginine and glycine, since a deficiency of these amino acids causes the occurrence of symptoms in the chick which resemble closely those observed in vitamin B₄ deficiency.

3 Vitamin B₅

The term vitamin B₅ was given in 1930¹¹ to a fuller's earth eluate fraction from yeast which was shown to be a factor essential for maintaining weight in pigeons. Vitamin B₅ was found to be stable toward heat and alkali. Later investigations have shown¹² that the vitamin B₅ concentrate contains vitamin B₆ which latter compound has weight maintaining properties for the pigeon, but the effects of which are less pronounced than those of the vitamin B₅ concentrate. The remaining factor is now called vitamin B₅. It seems probable that vitamin B₅ is identical with nicotinic acid¹² since nicotinic acid exhibits the weight maintaining properties of vitamin B₅ and has been isolated from eluate fractions.¹³ It has been reported independently that nicotinic acid is required by the pigeon.¹⁴

4 Vitamin B₇—Vitamin I

Vitamin B₇ or Vitamin I is a name given to a substance present in rice polishings which is soluble in methanol and in ethyl alcohol. In the absence of this factor, pigeons develop digestive disturbances.¹⁵

5 Vitamin B₈—Adenylic Acid

It has repeatedly been suggested¹⁶ that adenylic acid (or its degradation product, adenine) might exert vitamin activity and the term vitamin B₈

⁸ O. L. Kline, H. R. Bird, C. A. Elvehjem and E. B. Hart, *J. Nutrition* 11, 515 (1936); 12, 455 (1936).

⁹ V. Reader, *Biochem. J.* 24, 187 (1930).

¹⁰ D. M. Hegsted, G. M. Briggs, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.* 140, 701 (1941).

¹¹ C. W. Carter, H. W. K. Unersky and R. A. Peters, *Biochem. J.* 24, 1832, 1844 (1930).

¹² C. W. Carter and J. R. O'Brien, *Ibid.* 33, 1810 (1939).

¹³ T. F. Macrae and C. E. Edgar, *Ibid.* 31, 2225 (1937).

¹⁴ L. J. Harris, *J. Soc. Chem. Ind.* 58, 471 (1939).

¹⁵ E. Centanni, *Biochim. terap. sper.* 22, 137 (1935).

¹⁶ H. v. Euler, F. Schlenk, L. Melzer and B. Höglberg, *Z. phys. Chem.* 258, 212 (1939).

has been assigned to this compound. Adenine has been shown to be necessary for certain strains of lactic acid bacteria¹⁷ and it has been claimed that adenylic acid is the coenzyme of the fatty acid dehydrogenase¹⁸. In clinical studies¹⁹ on pellagrins adenylic acid seemed to increase the effect of nicotinic acid. When 3 to 20 mg of yeast adenylic acid were injected intravenously into patients deep involuntary, gasping inspiration, a fluttering sensation in the upper part of the abdomen and a feeling of fullness in the head were observed.

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¹⁷ E. F. Möller *Z. angew. Chem.* 52: 466 (1939).

¹⁸ E. Lang and H. Meyer *Z. physiol. Chem.* 262: 120 (1939).

¹⁹ T. D. Spies, D. P. Hightower and L. H. Hubbard *J. Am. Med. Assoc.* 115: 297 (1940).

²⁰ A. G. Hogan and E. M. Parrott *J. Biol. Chem.* 132: 507 (1940).

²¹ A. G. Hogan, N. B. Guerrant and H. L. Kempster *Ibid.* 64: 113 (1925). A. G. Hogan and C. L. Shrewsbury *J. Nutrition* 3: 39 (1930). A. G. Hogan, L. R. Richardson and H. Patrik *Ibid.* 19: Proc. 14 (1940).

²² H. S. Wiggus, I. C. Norris and G. I. Heller *J. Nutrition* 14: 14 (1937).

²³ T. H. Jukes *J. Biol. Chem.* 134: 83 (1940).

²⁴ A. G. Hogan, L. R. Richardson, H. Patrik and H. L. Kempster *J. Nutrition* 21: 327 (1941).

²⁵ H. v. Euler, H. Söder and M. Malmberg *Z. Hyg. i. f. d. Infektionskrankh.* 116: 677 (1935).

postulated because guinea pigs infected with pneumococci show remarkable resistance when lemon juice is administered. Since it is known that vitamin C is present in lemon juice and since vitamin C is known to detoxify bacteria, the power of ascorbic acid for protecting guinea pigs against pneumonia was studied experimentally. The effect of ascorbic acid was found to be considerably less than that of lemon juice. It was therefore concluded that a special anti pneumonia factor exists in lemon juice. As a further differentiation from vitamin C, vitamin J does not occur in paprika and in the eye lens. In addition to lemons, it is also found in black currants, rowanberries and elderberries. The chemical nature of vitamin J is not known. No studies have been published on the use of this vitamin in man.

9 Vitamins L_1 and L_2 —Lactation Vitamins

The existence of two different water soluble vitamins, necessary for the onset of normal lactation, has been postulated.²⁶ Vitamin L_1 is present in liver while vitamin L_2 is present in baker's yeast. Neither of these factors can replace the other. Much higher amounts of these vitamins are apparently necessary to induce the first lactation than are necessary for a second litter. The vitamins seem to be of functional importance in the maturation of lactation tissue.

Evidence for the existence of a factor essential for lactation has also been presented by another laboratory.²⁷ The factor was found in rice polishings, defatted wheat embryo and brewer's yeast, but most abundantly in liver and in rice bran extracts.

The factor or factors necessary for lactation appear to be different from all other known vitamins.^{26, 27, 28}

10 Vitamin M

The existence of a vitamin M⁹ has been postulated, because monkeys kept on a diet supposedly containing all recognized food factors are known

²⁶ W. Nakahara, F. Inukai and S. Ugami, *Science* 87: 372 (1938); *Sci. Papers Inst. Phys. Chem. Research Tokyo* 31: 42 (1937); 34: 250 (1938); *Proc. Imp. Acad. Tokyo* 14: 9 (1938).

²⁷ B. Sure, *J. Biol. Chem.* 140: CXXIX (1941).

²⁸ W. Nakahara, F. Inukai and S. Ugami, *Science* 91: 431 (1940).

⁹ P. L. Day, W. C. Langston and W. J. Darby, *Proc. Soc. Exptl. Biol. Med.* 38: 860 (1938); W. C. Langston, W. J. Darby, C. F. Shukers and P. L. Day, *J. Exptl. Med.* 68: 923 (1938); P. L. Day, W. C. Langston, W. J. Darby, J. C. Wahlin and V. N. M., *Ibid.* 72: 463 (1940); M. Janota and G. M. D., *Am. J. Infectious Diseases* 65: 217 (1939).

to suffer from anemia, cytopenia and loss of weight often accompanied by ulceration of the gums and by diarrhea. Monkeys on a diet devoid of the postulated vitamin M die in 26 to 100 days. The vitamin is present in yeast and in liver and is therefore probably a member of the vitamin B complex. Vitamin M deficiency causes a lowered microbic resistance in the gastrointestinal mucosa, and gingivitis and ulcerative colitis result from the action of pathogenic saprophytes (for example, *Bacterium dysenteriae* (Flesner), *Shiga bacillus*, etc.) normally present in the intestines. There is a possibility³⁰ that a nutritional deficiency is also an essential etiological factor in human dysentery.

11 Factor T

The existence of a fat soluble factor T has been postulated.³¹ The absence of this factor causes a decrease in the number of blood platelets (thrombocytosis) in rats and man. Factor T is reported to occur in sesame oil and in egg yolks, but not in cod liver oil or olive oil. The chemical nature of this compound is not known, but it has been observed that its activity is destroyed by ultraviolet irradiation.

12 Factor U

Factor U is a growth promoting substance required by chicks.^{32, 33} It is present in yeast, wheat bran middlings, alfalfa meal and to a lesser extent, in corn. The factor is insoluble in ether, acetone and isopropyl alcohol, but soluble in 50 per cent methanol and can be adsorbed on fuller's earth and charcoal. The factor is stable in yeast when autoclaved for half an hour at pH 1.7 or 11.0 but is destroyed in alfalfa when autoclaved for five hours at 120° C.

13 Folic Acid

Folic acid³⁴ (derived from the latin *folium*—leaf) occurs in green leaves of many kinds including grass. Mushrooms and yeast are good sources. It is also present in a number of animal tissues of which liver and kidney are the best sources.

³⁰ G. F. McGunes, A. L. McLean, F. Spindler and K. F. Maxcy, *Am. J. Hyg.* **24**, 552 (1938). Editorial, *J. Am. Med. Assoc.* **116**, 2169 (1941).

³¹ E. Schaff and C. Hirschberger, *Am. J. Diseases Child.* **53**, 32 (1937). *Jahrb. Kinderheilk.* **146** [N. F. 96] 181, 191, 93 (1936). 147 [N. F. 97] 81 (1936).

³² E. L. R. Stokstad and P. D. V. Manning, *J. Biol. Chem.* **125**, 687 (1938). *Science* **88**, 35 (1939).

³³ E. L. R. Stokstad, P. D. V. Manning and R. E. Rogers, *J. Biol. Chem.* **132**, 463 (1940).

³⁴ H. K. Metcalf, E. R. Snell and R. J. Williams, *J. Am. Chem. Soc.* **63**, 284 (1941).

Folic acid has been isolated from spinach by adsorption on charcoal, precipitation with lead and silver salts and chromatographic adsorption on fuller's earth. Folic acid has a molecular weight of about 500, contains nitrogen but no phosphorus or sulfur, and is acidic in character.

Folic acid stimulates growth of rats and of various bacteria including *Streptococcus lactis* R, *Lactobacillus delbrueckii* and *Lactobacillus casei*.³⁵

14 Growth Factor for *Lactobacillus casei*

The existence of a growth factor for *Lactobacillus casei* was demonstrated by the same method which was used for the isolation of folic acid (see page 525). The chemical characteristics of the growth factor are, however, different from those of folic acid. This factor was isolated³⁶ from liver by adsorption on norite, followed by elution with ammonium hydroxide in methanol solution, followed by fractional precipitation with manganese salts. The product obtained has the properties of a dinucleotide containing a purine and a pyrimidine nucleus. Guanine was isolated from the hydrolysis products.

15 Grass Juice Factor

The existence of a special factor necessary for optimal growth of rats³⁷ and of guinea pigs^{38, 39, 40} has been postulated. This factor is present in fresh grass³⁷ and in its juice³⁸ and has therefore been called grass juice factor. Cereal grasses, rye grass, young white clover, peas, pea shells, cabbage, turnip tops and spinach are excellent sources. Young berries, cauliflower, canned peas and beans contain less. Relatively little of this factor is present in apples, celery, molasses, peanuts, turnips, lettuce and oats.⁴¹ Animal materials such as liver contain only small quantities. The amount present in grass varies with the age of the plant, the maximum concentration being present in the growing plant while the mature and old plants contain considerably less.^{38, 41} The active material can be preserved in forages by careful ensiling.⁴ Acid methods of ensiling are superior

³⁵ E. E. Snell and W. H. Peterson *J. Biol. Chem.* 39: 273 (1940).

³⁶ E. L. R. Stokstad *J. Biol. Chem.* 139: 475 (1941).

³⁷ G. O. Kohler, C. A. Elvehjem and E. B. Hart *J. Nutrition* 14: 131 (1937).

³⁸ G. O. Kohler, C. A. Elvehjem and E. B. Hart *Ibid.* 15: 445 (1938).

³⁹ M. D. Cannon and G. A. Emerson *Ibid.* 18: 155 (1939).

⁴⁰ G. O. Kohler, S. B. Randle, C. A. Elvehjem and E. B. Hart *Proc. Soc. Exptl. Biol. Med.* 40: 154 (1939).

⁴¹ S. B. Randle, H. A. Sober and G. O. Kohler *J. Nutrition* 20: 459 (1940).

⁴² E. C. Johnson, C. A. Elvehjem, W. H. Peterson and H. J. Fagen *Ibid.* 18: 527 (1939).

to other methods especially to the molasses method and the dry method. The factor is destroyed by heat and by oxidation ⁴¹

The 'grass juice factor' appears to be different from all other known vitamins. It can be precipitated from grass juice with acetone and then removed from this precipitate by means of acidified acetone. When grass juice is deproteinized with chloroform and alcohol, the factor remains in the water phase and can be adsorbed on norite ⁴²

The grass juice factor is apparently readily absorbed by experimental animals and is secreted into milk ³⁷ ⁴³. Therefore the summer milk of cows contains considerable amounts of this factor while winter milk is practically devoid of it.

In the absence of the grass juice factor rats and guinea pigs show a decline in weight and finally die. The rat is less sensitive to a deficiency than the herbivorous guinea pig. In addition to the loss in weight, some respiratory trouble was noted in most cases. The lungs show inflammation and congestion and in isolated cases necrotic areas were observed ³⁸

16 Mouse Factor

Mice on a synthetic diet but not on stock diets, have been shown ⁴⁴ to develop sore eyes, uni- or bilateral, characterized by swelling and inflammation of the eyelids leading to closure of the eyes and blindness in many cases. Simultaneously a dermatitis on the ventral aspect of the neck between the forelegs and extending almost up to the lower lip, and some loss of hair, especially on the face and neck occur. Finally the animals die. Rats on the other hand do not develop this disease when kept on the same diet. The symptoms can be cured by normal diet. Aqueous liver extracts and fresh flaked wheat germs are especially potent cures. In many cases the symptoms could not be cured completely suggesting that the damage if too extensive is irreparable.

17 Anti-Pernicious Anemia Factor

There is the possibility that the postulated extrinsic factor in the absence of which pernicious anemia occurs in man will eventually be found to be a vitamin. Little is known about this factor. In the presence of an intrinsic factor the extrinsic factor is supposedly converted into the compound which prevents the onset of pernicious anemia. The latter com-

⁴¹ G. O. Kohler, S. B. Randle and J. R. Wagner, *J. Biol. Chem.* 128 LV (1939)

⁴² C. Carruthers, *Science* 93 44 (1941)

pound is found predominantly in the liver, but the natural distribution of the extrinsic factor and its chemical characteristics are unknown. This is due to the fact that it has been impossible to reproduce the human macrocytic hyperchromic anemia in animals.

APPENDIX
THE VITAGENS

THE VITAGENS

ESSENTIAL FATTY ACIDS

1 Nomenclature and Survey

Names

Essential fatty acids

Vitamin F^{1, 2}

Vitamin F₁

List of known essential fatty acids

Linoleic acid $C_{18}H_{32}O_2$

Linolenic acid $C_{18}H_{30}O_2$

Arachidonic acid $C_{20}H_{38}O_2$

Formulas

Linoleic acid



Linolenic acid



Arachidonic acid



2 Chronology

- 1929 G O BURR and M M BURR³ showed that the presence of highly unsaturated acids such as linoleic and linolenic acid in the diet is essential for normal growth of rats
- 1938 TURPEINEN⁴ found arachidonic acid more potent than any other of the investigated unsaturated acids and introduced the tentative hypothesis that the body needs this acid primarily NUNN and SMIDLEY MACLEAN⁵ established that arachidonic acid was deposited in the liver fat when linoleic acid was fed to rats

¹ H M Evans S Lepkovsky and E A Murphy *J Biol Chem* 106 431 (1934)

² The Council on Pharmacy and Chemistry of the American Medical Association the American Society of Biological Chemists and the American Institute of Nutrition recommended that the term vitamin F should not be used in referring to linoleic or linolenic acids or any fatty acid or mixture of fatty acids *J Am Med Assoc* 113 589 (1939)

³ G O Burr and M M Burr *J Biol Chem* 82 345 (1929) 86 618 (1930)

⁴ O Turpeinen *J Nutrition* 15 351 (1938)

⁵ L C A Nunn and I Stedley MacLean *Biochem J* 32 2178 (1938)

3 The Essential Fatty Acids as Vitagens

Much controversy has arisen over the question of whether or not the essential fatty acids should be classified as vitamins. In view of the definition of vitagens, as given on pages 5-6, the essential fatty acids are vitagens, since they are needed only in small quantities (0.1% of the food) and since they contribute to the mechanism of the transformation of energy, but are also building units of the phospholipides.

Which of the fatty acids are really essential is not known. The early work was done with linoleic and linolenic acids, both of which are able to counteract the deficiency symptoms, but apparently neither of these is stable in the organism. It has, however, been demonstrated⁸ that after an intake of linoleic or linolenic acid, arachidonic acid was deposited in the liver fats. This may suggest that linoleic and linolenic acids are active precursors, whereas arachidonic acid is physiologically the most important compound. There are quite a number of other highly unsaturated fatty acids which occur naturally, but their vitagen nature has not been investigated as yet. In the following pages only the acids with known vitagen character will be discussed.

4 Occurrence

The essential fatty acids occur naturally both in plants and in animals. For a consideration of their occurrence, each fatty acid must be studied separately.

1 *Linoleic acid* occurs as a glyceride in most drying oils such as cotton seed, poppy seed, corn⁶ and other oils. In animals, linoleic acid is present chiefly in the fatty acids of the phospholipides and to a lesser extent in the neutral fat.

2 *Linolenic acid*, like linoleic acid, occurs as a glyceride in most drying oils, such as linseed and perilla oils. Linolenic acid is usually absent from animal fats.

3 *Arachidonic acid* occurs predominantly in animal tissues. It is found mainly in the phospholipide fraction as a constituent of lecithin and cephalin,⁷ but occurs also as a constituent of neutral fat. This acid has been isolated from heart⁸, spleen,⁸ liver,⁹ adrenal⁸ and brain^{10, 11} fatty acids.

⁶ T. C. Taylor and J. M. Nelson *J. Am. Chem. Soc.* 42 1726 (1920)

⁷ P. A. Levene and I. P. Rolf *J. Biol. Chem.* 51 507 (1922) 54 91 99 (1922)

⁸ E. Fleck and O. von Schoenebeck *Z. physiol. Chem.* 209 112 (1932)

⁹ P. Hartley *J. Physiol.* 38 353 (1900)

¹⁰ E. Klenk and J. Dittmar *Z. physiol. Chem.* 244 203 (1936)

¹¹ D. Wesson *J. Biol. Chem.* 60 183 (1924)

5 Isolation

The isolation of the essential fatty acids comprises the isolation of the total fatty acids from plant or animal materials followed by the separation of the individual fatty acids

Plant material is either saponified as such or the oil present in plants, such as in seeds, is pressed out first, and then saponified. Alkali or alkaline earth hydroxide is usually used for these saponifications. The acids are usually extracted from the saponification mixture with an organic solvent. Linoleic and linolenic acids are separated from the other fatty acids by the addition of bromine whereby the tetra- and the hexabromide respectively, precipitate. The brominated acids are separated and debrominated by means of zinc and alcohol. Thus a mixture of linoleic and linolenic acids is obtained which can be separated by means of the different solubilities of their zinc salts in alcohol.

Animal materials are either saponified as such or the total fat is extracted or separated into the phospholipides and true fats. The latter procedure is carried out by dehydrating the material either by a drying process or better by means of acetone. The total fatty material is then extracted with alcohol preferably at elevated temperature and in the presence of inert gases such as nitrogen. After removal of the solvent, the phospholipide fraction is precipitated with acetone while the true fats go into solution. This division into neutral fats and phospholipides is not a quantitative procedure, but serves for a gross separation. The fractions are then saponified and worked up as described for plant material. Upon bromination, the octabromide of arachidonic acid is obtained which yields arachidonic acid upon debromination.

The yields of the unsaturated acids obtained by the bromination debromination procedure are rather low, but the purity is considered to be high, at least in the case of linoleic and linolenic acids. To a certain extent, however, these acids and especially arachidonic acid undergo a rearrangement of the position of the double bonds.

A principally different method can be used for the isolation of the unsaturated fatty acids namely, the direct isolation by fractional crystallization at low temperature. The yields by this method are high but the purity of the product is not as satisfactory. Practically, the phosphatide fraction is used for the isolation and the phosphatides are converted into methyl esters by direct alcoholysis.¹² Fractional crystallization is then carried out at various low temperatures for example, consecutively at -20° ,

¹² G. Y. Shinowara and J. B. Brown, *Oil & Soap* 15 151 (1938).

-40° and -80° C By this method linoleic acid has been obtained from cottonseed and from corn oils in 90-94% purity,¹³ linolenic acid from linseed and from perilla oils in 85-88% purity¹⁴ and arachidonic acid from adrenal phosphatides in 70-75% purity¹⁵ Further purification, for example, of the methyl arachidonate, can be effected by fractional distillation, whereby a product of 95% purity is obtained¹⁶

6 Properties

All essential fatty acids have similar solubility characteristics They are insoluble in water, but soluble in alkali and in organic solvents

- (1) Linoleic acid Colorless liquid at room temperature M p 11° C
- (2) Linolenic acid Colorless liquid B p $230-232^{\circ}$ C /17 mm Hg
- (3) Arachidonic acid Colorless crystals M p 77° C

7 Chemical Constitution

All essential fatty acids are carboxylic acids as evident from the formation of various salts, analytical data and the formation of various esters

1 *Linoleic acid* has the empirical formula $C_{18}H_{32}O_2$ and contains two double bonds as evident from the formation of a tetrabromide and of tetrahydroxy stearic acid (sativinic acid) upon mild oxidation with permanganate¹⁶ Other oxidation products are azelaic acid, oxalic acid and *n* caproic acid¹⁷ Upon reduction with hydriodic acid and phosphorus, stearic acid is obtained¹⁸ Linoleic acid is therefore, $\Delta^9, 12$ octadecadienoic acid



2 *Linolenic acid* has the empirical formula $C_{18}H_{30}O_2$ and contains three double bonds since upon addition of bromine a hexabromo derivative is formed Upon reduction stearic acid is obtained Linolenic acid is therefore a straight chain fatty acid¹⁹ Oxidation with ozone yields propionaldehyde, malonic aldehyde carboxylic acid and azelaic aldehyde

¹³ J B Brown and G C Stoner *J Am Chem Soc* 59 3 (1937) J B Brown and J Frankel *Ibid* 60 54 (1938)

¹⁴ G Y Shinowara and J B Brown *Ibid* 60 2734 (19 8)

¹⁵ G Y Shinowara and J B Brown *J Biol Chem* 134 331 (1940)

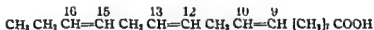
¹⁶ A Rollett *Z physiol Chem* 62 410 (1909)

¹⁷ G L Goldsobel *Chem Ztg* 30 825 (1906)

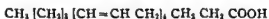
¹⁸ K Peters *Monatsh* 7 502 (1886)

¹⁹ F Erdmann and F Bedford *Ber* 42, 1324 (1909)

carboxylic acid The three double bonds of linolenic acid are therefore in Δ 9 12 15 positions according to the formula



3 *Arachidonic acid* has the empirical formula $\text{C}_{20}\text{H}_{32}\text{O}_2$ and contains four double bonds An octabromo arachidonic acid is formed upon bromination The straight chain structure is established by total hydrogenation and comparison with a synthetic π eicosanic acid²⁰ The position of the four double bonds has been established by ozonolysis which yielded caproic, acetic succinic, malonic and glutaric acids, acetaldehyde and carbon dioxide Arachidonic acid therefore has been assigned the structure of Δ 5 8 11, 14 eicosa tetrenoic acid^{20a}



An eicosa tetrenoic acid which is apparently different from arachidonic acid, has been isolated from sardine oils This acid has the double bonds in 4, 8 12 and 16 positions since upon oxidation succinic and butyric acids exclusively are obtained in almost quantitative yields²¹ The biological efficacy of this acid has not been investigated as yet

8 Synthesis

None of the essential fatty acids has been synthesized

9 Biogenesis

The origin and the biogenesis of linoleic acid and of linolenic acid in plant material are largely unknown It has been speculated that certain plants may contain a dehydrogenation mechanism whereby saturated fatty acids are converted into unsaturated fatty acids However it is just as probable that the unsaturated fatty acids are obtained by total synthesis as is the case of the saturated fatty acids

A better insight exists concerning the biogenesis of arachidonic acid Upon feeding of linoleic or linolenic acid to rats arachidonic acid is deposited in the tissues²² This proves a partial synthesis of this acid

²⁰ G. Y. Shinowara and J. B. Brown *J. Biol. Chem.* 134 331 (1940)

^{20a} D. E. Dolby, J. C. A. Nunn and I. Smedley MacLean *Biochem. J.* 34 1472 (1940) D. T. Mowry, W. B. Brode and J. B. Brown *J. Biol. Chem.* 142 679 (1947) C. J. Arcus and I. Smedley MacLean *Biochem. J.* 37 1 (1943)

²¹ Y. Toyama and T. Tsuchiya *Bull. Chem. Soc. Japan* 10 296 (1933)

²² L. C. A. Nunn and I. Smedley MacLean *Biochem. J.* 32 2178 (1938)

in the organism of the rat and indicates that rats are incapable of synthesizing a precursor of arachidonic acid

10 Specificity

It has already been mentioned that of all the fatty acids investigated, only linoleic, linolenic and arachidonic acids show activity. Besides the free acids, the esters are active. The esters of the lower aliphatic alcohols have been demonstrated to be active and it is expected that all esters which can be hydrolyzed in the organism will prove effective.

The relative efficacy of the active acids, tested as their methanol esters, is recorded in the following table showing the symptom efficacy according to the skin test and growth test on a comparative basis arbitrarily designating the activity of linoleic acid in both tests as 1.

Acid	Efficacy in skin test	Efficacy in growth test
Arachidonic acid ²³	1	6
Linoleic acid	1	1
Linolenic acid	$\frac{1}{4}$	$\frac{1}{4}$
Docosa hexaenoic acid ($C_{22}H_{42}O_2$) ²⁴	0	Little
Linusic and isolinusic acid	0	0

Tetrahydroxy stearic acids, dioxido stearic acid, 9,10,12 trihydroxy stearic acid and chaulmoogric acid are inactive.

11 Determination

(a) Chemical Methods

The only known chemical method for the determination of the essential fatty acids is the precipitation of their bromine addition compounds which has been described on page 533.

A number of color reactions have been proposed² for the determination of the essential fatty acids, which are based upon reactions of the double bonds. Thus the transfer of hydrogen from aromatic amines such as *p* phenylene diamine either alone or in the presence of phenols, for example, *o* cresol, to the essential fatty acids has been studied. The colors developed depend upon the concentration used and the time. Initially a violet then

²³ E. M. Hume, L. C. A. Nunn, I. Smedley MacLean and H. H. Smith, *Biochem. J.* **34**, 879 (1940).

²⁴ E. H. Farmer and F. A. Van de Heuvel, *J. Chem. Soc.* 1938, 427.

²⁵ G. Woker and P. Bernhard, *Helv. Chim. Acta* **24**, 98 (1941).

an olive green and finally a gray color are observed. This reaction can be made considerably more sensitive by the addition of hydrogen peroxide. Another reaction which has been studied, is the addition of iodine to the double bonds. All these reactions are, however, not specific for the essential fatty acids since they are given by other unsaturated but non-essential fatty acids by carotenoids, etc.

(b) *Biological Methods*

(a) **The Rat Growth Method** ²⁵ This method led to the discovery of the essential fatty acids and can be used either prophylactically or as a curative method and is based on increase in weight following the feeding of the essential fatty acids to young rats which have been depleted of these acids. The depletion of this factor is a very slow process and may take many months.

(b) **The Rat Skin Test** ²⁷ In this curative method, the criterion is the healing of the characteristic dryness and scurfiness on the dorsal surface of the hind feet and front of the ankles. Instead of feeding the essential fatty acid preparation, the material may also be applied topically to the skin. ²⁸

(c) **The Oestrus Cycle Test** ²⁹ Irregular ovulation observed during essential fatty acid deficiency, has been used for the determination of the deficiency of these factors and the restoration of the oestrus cycle toward normal has been used for the determination of the presence of the essential fatty acids.

12 Standards

There is no recognized standard fatty acid for comparison of its biological effect with that of unknown material. Since it is desirable to set up such a standard reference essential fatty acid, it is recommended that arachidonic acid be adopted, since all available evidence indicates that this is the acid which is produced in the organism from the other known essential acids.

13 Physiology

Little is known about the physiology and the mode of action of the essential fatty acids. Practically all experimental work has been carried out with rats.

²⁵ G. O. Burr and M. M. Burr, *J. Biol. Chem.* 82: 345 (1929); 86: 618 (1930).

²⁷ E. M. Hume, L. C. A. Nunn, I. Smedley, MacLean and H. H. Smith, *Biochem. J.* 32: 16 (1938).

²⁸ M. I. Shepherd and D. R. Linn, *Drug Cosmetol.* 1: 38: 679 (1936).

²⁹ O. Turpeinen, *J. Nutrition*, 15: 351 (1939).

Linoleic and linolenic acids and their simple esters are absorbed from the intestinal tract. Linolenic acid is metabolized directly after entering the organism and is not stored as such unless it is administered in excessive doses³⁰. Linoleic acid, on the other hand, has been found invariably in all investigated animals and in man in the fatty acids of the phospholipides and to a lesser extent in the neutral fats. The main unsaturated acid in the phospholipides is arachidonic acid. The essential fatty acids are held in the phospholipides with extreme tenacity³¹ and it takes therefore several months before a rat is completely depleted of these acids.

It is possible that linoleic and linolenic acids are really precursors of the active form. Linolenic acid cannot be demonstrated to be present in the organism although its biological efficacy is established. Linoleic and linolenic acids are converted in the organism into arachidonic acid^{32, 33} which is the most active acid known. Besides arachidonic acid, other highly unsaturated fatty acids such as an acid $C_{26}H_{44}O_2$, dihydro arachidonic acid, docosa penta enoic acid, etc., are synthesized in the body from linoleic and linolenic acids. Whether or not some of these act as "essential fatty acids" is not known.

The mechanism of the action of the essential fatty acids is not quite clear. Apparently these acids act in phosphatides and take part in the mechanism of utilizing fats. It has been suggested³⁴ that the first action is to load up the connective tissue cells of the fat depots with fats. If only small amounts of the essential fatty acids are administered this effect can be demonstrated separately. After the depots are filled up with fat and additional amounts of the essential fatty acids are offered, growth takes place. Simultaneously the excessive fat deposits disappear.

The essential fatty acids are, however, not only connected with the movement of fats but also with the utilization of fats. It has been shown in *in vitro* experiments that unsaturated fatty acids catalyze the oxidation of saturated fatty acids without being affected themselves.

From experiments with fat starved rats, the conclusion was drawn³⁵ that the essential fatty acids are necessary for the formation of new tissue but not for the maintenance of the normal metabolism of the cell.

³⁰ N. R. Ellis and H. S. Isbell *J Biol Chem* 69 219 (1928). R. Klenk *Z physiol Chem* 206 95 (1932). R. H. Snider and W. R. Bloor *J Biol Chem* 99 555 (1933).

³¹ R. G. Sinclair *Proc Soc Exptl Biol Med* 27 1059 (1930).

³² N. R. Ellis and H. S. Isbell *J Biol Chem* 69 219 (1920). H. C. Eckstein *Ibid* 81 613 (1929).

³³ J. M. Spadola and N. R. Ellis *Ibid* 113 205 (1936).

³⁴ L. C. A. Nunn and I. Smedley MacLean *Biochem J* 32 2178 (1938).

³⁵ I. Smedley MacLean and L. C. A. Nunn *Ibid* 34 884 (1940).

³⁶ I. Smedley MacLean and E. M. Hume *Ibid* 35 990 (1941).

14 Deficiency Syndrome

A deficiency of the essential fatty acids causes a typical dermatosis in rats characterized by general dryness of the skin and thinness with much scurf especially on both the fore and hind paws and on the ears and tail. The latter may appear to be annulated. It should be noted that there is no edema present as is in the otherwise quite similar symptoms caused by a vitamin B₆ deficiency. In fat deficiency the lacrimal glands are also affected and irregular ovulation and lesions of the kidney and of the urinary tract occur. Abnormal reproduction and deficient lactation have been observed. In young rats cessation of increase in length and weight occurs. Sexual development is also delayed.

Clinical symptoms of a deficiency of the essential fatty acids in man are not known. There are, however, some observations which indicate that in certain eczema, and especially in infantile eczema, favorable clinical results can be obtained when oils containing unsaturated fatty acids are administered.³⁶ In such pathological cases it was found that the fatty acids from the blood serum contained a significantly lower concentration of unsaturated compounds than those of healthy individuals. By the time a clinical improvement was noticed, the concentration of unsaturated fatty acids had risen to essentially normal levels.

(a) Clinical Test Methods

There are no accurate methods for determining a deficiency of the essential fatty acids. It has been proposed to assay the actual concentration of unsaturated fatty acids in the serum since it was found that in rats,³⁷ goats³⁸ and dogs³⁹ the degree of unsaturation diminished with a lowered intake of the unsaturated fatty acids. These methods, however, have not been perfected and do not permit a differentiation of the essential fatty acids from non essential, unsaturated fatty acids.

15 Requirements

The requirements of the essential fatty acids are largely unknown. In rats, a daily intake of about 14 mg methyl arachidonate produces optimum effects⁴⁰ but decreased amounts show less favorable responses.

³⁶ A. E. Hansen *Proc Soc Exptl Biol Med* 30 1198 (1933) 31 160 161 (1933) T. Cornbleet *Arch Dermatol Syphilol* 31 224 (1934)

³⁷ A. E. Hansen and G. O. Burr *Proc Soc Exptl Biol Med* 30 1 00 1201 (1933)

³⁸ H. H. Williams and L. A. Maynard *J Dairy Sci* 17 223 (1934)

³⁹ A. E. Hansen, W. R. Wilson and H. H. Williams *J Biol Chem* 114 209 (1936)

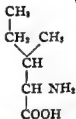
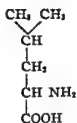
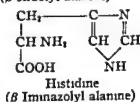
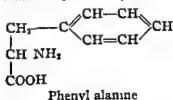
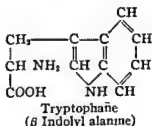
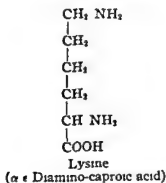
⁴⁰ E. M. Hume, L. C. A. Nunn, I. Smedley MacLean and H. H. Smith *Biochem J* 34 879 (1940)

ESSENTIAL AMINO-ACIDS

Among the amino acids which are normal constituents of dietary proteins, ten are essential. Those amino acids are defined^{1, 2} as "essential" which must be present in the diet of young rats, in addition to all other energy and building unit requisites and the vitamins, to insure optimal growth. That is, these amino acids cannot be synthesized in the animal organism or at least not at a rate necessary for normal growth. These ten amino acids are^{1, 2} lysine, tryptophane, histidine, phenyl alanine, leucine, isoleucine, threonine, methionine, valine, arginine.

A deficiency of any one or of all of these ten amino acids causes an inhibitory effect upon growth. The same amino acids which are essential for rats are also essential for dogs.³ It is reasonable to assume that they are also indispensable for the human body, but no definite information regarding the special amino acid requirements of man is available.

Arginine holds a somewhat unique position. Rats on an arginine free diet are able to synthesize some of their requirements since the animals show a



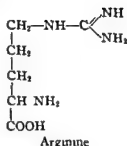
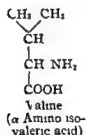
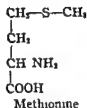
Leucine (α Amino
iso-caproic acid)

Isoleucine (α Amino- β methyl valeric acid)

Threonine (α Amino- β hydroxy butyric acid)

¹ W C ROSE *Physiol Rev* 18 109 (1938) ² W C ROSE *Science* 86 298 (1937)

W C Rose and E E Rice *Ibid* 90 186 (1939)



growth of 70–80% in comparison with animals which received arginine⁴. In mice the synthesis of arginine from ornithine has been demonstrated by means of labeling the compounds with deuterium⁵. Adult dogs can get along without an external supply of this amino acid. Chicks, on the other hand, must rely upon dietary arginine⁶ since this amino acid is needed as a structural building unit of creatine for feather formation.

While retarded growth is a somewhat non specific symptom for a vitamin or vitagen deficiency specific deficiency symptoms are known for a few of the indispensable amino acids. The fright disease of dogs has for example, been correlated with inadequate amino acid supply⁷. A deficiency of lysine causes anemia in rats⁸. Cataract and other ocular changes have been observed in young rats on a tryptophane deficient diet⁹. Rats deprived of valine show a disorder of the nervous system characterized by sensitivity to touch and severe lack of coordination in movement¹⁰. Young chicks fed a diet devoid of arginine and glycine develop a typical paralysis⁶. For the effects caused by a deficiency in methionine see pages 543 and 549. Other specific deficiency syndromes will undoubtedly be observed upon further studies.

The exact requirements of the essential amino acids for man and various animal species are not known. The minimum amount necessary to support normal growth has been determined only for rats¹⁰. The total amount of essential amino acids corresponds to 5.8% of the food consumed with the following figures for the individual amino acids: lysine 1.0%, leucine 0.9%, phenylalanine 0.7%, valine 0.7%, threonine 0.6%, methionine 0.6%, isoleucine 0.5%, histidine 0.4%, tryptophane 0.2% and arginine 0.2%.

⁴ C. W. Scull and W. C. Rose *J Biol Chem* 89 109 (1930)

⁵ R. F. Clutton, R. Schoenheimer and D. Rittenberg *Ibid* 132 227 (1940)

⁶ A. Arnold, C. L. Kline, C. A. Elvehjem and E. B. Hart *Ibid* 116 699 (1936) D. M. Hegsted

G. M. Briggs, C. A. Elvehjem and E. B. Hart *Ibid* 140 191 (1941)

⁸ A. Arnold and C. A. Elvehjem *J Am Vet Med Assoc* 95 303 (1939)

⁹ A. G. Hogan, E. L. Powell and R. E. Guarrant *J Biol Chem* 137 41 (1941)

¹⁰ J. R. Totter and P. L. Day *Ibid* 140 CXXXIV (1941)

¹¹ W. C. Rose *Science* 86 298 (1937)

ESSENTIAL CARBOHYDRATES

In analogy to the discovery that besides the energy bearing and building unit supplying compounds of the class of fats and proteins there are some fats and proteins which are also protective food constituents and which are therefore classified as vitagens, there may be specific carbohydrates which are vitagens. Ascorbic acid and inositol are examples of essential carbohydrates which are vitamins. Whether or not other carbohydrates exist which are essential food constituents is not known. If carbohydrates as food constituents would be utilized only to supply energy, it should be possible to replace all carbohydrates with the exception of the carbohydrates of vitamin character by some other energy bearing foods such as fats or proteins. This is, however, not the case. The animal organism maintains, for example, in the blood, a certain normal glucose concentration and it has been shown that the organism is able to synthesize a certain amount of glucose for this purpose ^{1 2 3}. Animals on a completely carbohydrate free diet, however, do not survive, apparently because the amount of glucose synthesized is not sufficient to maintain the necessary carbohydrate concentration in the organism. It is thus possible that either carbohydrates in general or some specific carbohydrates may, eventually, be found to be essential protective food constituents.

There is some evidence for the existence of an additional growth factor of the carbohydrate class at least for the growing chick. This growth factor was first encountered in rice and has therefore been called 'rice factor'. This factor is apparently not present in yeast ⁴. It has also been found in cartilage and the effect has been shown to be caused by the glucuronic acid component of chondroitin ⁵. The effect does not appear to be very specific but is also brought about by a number of other carbohydrates, such as gum arabic, sodium alginate, gluconic acid, galactonic lactone, arabinose and xylose, while sugars like *d*-ribose and rhamnose are apparently inactive ⁶.

¹ R. D. Cramer and G. B. Kistiakowsky *J. Biol. Chem.* **137**: 540 (1941)

² J. B. Couant, R. D. Cramer, A. B. Hastings, F. W. Klemperer, A. K. Solomon and B. Vennesland *Ibid.* **137**: 557 (1941)

³ A. K. Solomon, B. Vennesland, F. W. Klemperer, J. M. Buchanan and A. B. Hastings *Ibid.* **140**: 171 (1941)

⁴ D. W. Hegsted, J. J. Oleson, C. A. Elvehjem and F. B. Hart *Poultry Sci.* **19**: 167 (1940). F. L. R. Stokstad, P. D. V. Manning and R. L. Rogers *Ibid.* **19**: 10, (1940)

⁵ H. J. Almquist, I. L. R. Stokstad, I. Meechi and P. D. V. Manning *J. Biol. Chem.* **134**: 213 (1940). H. J. Almquist, I. Meechi, I. L. R. Stokstad and I. D. V. Manning *Ibid.* **134**: 465 (1940)

⁶ E. L. R. Stokstad, H. J. Almquist, I. Meechi, I. D. V. Manning and R. L. Rogers *Ibid.* **137**: 373 (1941)

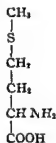
CHOLINE AND RELATED COMPOUNDS AND THE ESSENTIAL, TRANSFERABLE METHYL GROUP



Choline



Betaine



Methionine

1 Chronology

- 1894 HOFMEISTER¹ postulated the transfer of methyl groups in metabolism as the result of *in vivo* formation of methyl selenides and tellurides
- 1917 THOMPSON² concluded that the synthesis of creatine in the animal organism is the result of methyl transfer
- 1932 BEST and co-workers³ showed that in rats the addition of choline or betaine to a diet high in fat prevents the deposition of excess amounts of fats in the liver
- 1935 BEST and co-workers⁴ presented evidence that choline is an accessory food factor for rats
- 1937 TUCKER and ECKSTEIN⁵ found that methionine exerted an effect on liver fat similar to that of choline
- 1939 DU VIGNEAUD and co-workers⁶ observed that the essential amino acid methionine can be replaced in the diet of rats by homocysteine when choline or betaine is given simultaneously and suggested that this effect is due to the anabolic formation of methionine by methyl transfer
- 1940 BORSOOK and DUBNOFF⁷ and DU VIGNEAUD and co-workers⁸ proved that methyl transfer occurred in the synthesis of creatine in the animal organism. DU VIGNEAUD⁸ expressed the view that the transferable methyl group may be an essential dietary constituent

¹ F Hofmeister *Arch expil Path Pharmacol* 33 198 (1894)

² Thompson *J Physiol* 51 347 (1917)

³ C H Best J M Hershey and M E Huntsman *Am J Physiol* 101 7 (1932) *J Physiol* 75 56 (1932) C H Best and M E Huntsman *Ibid* 78 405 (1932)

⁴ C H Best M E Huntsman E W McHenry and J H Ridout *J Physiol* 84 38P (1935)

⁵ H F Tucker and H C Eckstein *J Biol Chem* 121 479 (1932) 126 117 (1938)

⁶ V du Vigneaud J P Chandler A W Moyer and D M Keppel *Ibid* 131 57 (1939)

⁷ H Borsook and J W Dubnoff *Ibid* 132 559 (1940)

⁸ V du Vigneaud J P Chandler M Cohen and G B Brown *Ibid* 134 787 (1940)

2 The Active Compounds and Their Properties

The number of naturally occurring compounds which may serve, among other purposes, as suppliers of the essential transferable methyl group is not known. However, besides choline, methionine and betaine are known to act in this capacity, especially in the absence of the former.

Choline Choline is a colorless viscid strongly alkaline liquid and is very hygroscopic. It absorbs carbon dioxide from the air and easily forms salts—for example the chloride borate picrate etc. Choline is very soluble in water and alcohol but is insoluble in ether. The salts are white hygroscopic crystals which are soluble in water and in alcohol. Their aqueous solutions are practically neutral.

Methionine M p 283° C under decomposition (Darkening at 278° C) $[\alpha]_D^{25} = -6.87^\circ$ C in water.

Betaine Betaine forms very hygroscopic crystals which lose their water at 100° C. Betaine melts at 293° C under rearrangement into the methyl ester of dimethyl amino acetic acid.

3 Pathological States Caused by a Deficiency of the Active Compounds

A deficiency of choline, betaine or methionine in the diet of young rats or dogs causes the deposition in the liver of fat⁹ and to a lesser extent also of cholesterol esters¹⁰. These are removed or their deposition prevented when choline or one of the other mentioned compounds is fed. These substances are called lipotropic factors. At the same time a diffuse nodular cirrhosis of the liver occurs and has been observed in rats,¹¹ dogs,¹² rabbits¹⁴ and guinea pigs¹⁵. The deficiency of these compounds further more causes hemorrhagic degeneration of the kidneys characterized by symmetrical hemorrhagic necrosis of the cortex¹⁶ ¹⁷ ¹⁸ ¹⁹. An involution of the thymus gland, an enlargement of the spleen, a transformation of the

⁹ C. H. Best, J. M. Hershey and M. E. Huntsman *Am J Physiol* 101:7 (1932); *J Physiol* 75:56 (1932); C. H. Best and M. E. Huntsman *Ibid* 75:405 (1932).

¹⁰ C. H. Best and J. H. Ridout *J Physiol* 78:415 (1933); 84:7P (1935); A. V. Strosser, I. McQuarrie and J. A. Anderson *Proc Soc Exptl Biol Med* 33:590 (1936); H. P. Himsworth *Acta Med Scand Supplement* 90:158 (1938).

¹¹ H. Blumberg *U S Pub Health Service Pub Health Repts* 55:334 (1940); H. Blumberg and H. G. Grady *Proc Am Soc Biol Chem* April 1941; H. Blumberg and E. V. McCollum *Science* 93:98 (1941); R. D. Lillie, F. S. Daft and W. H. Sebrell *Pub Health Rep* 56:1235 (1941); F. S. Daft, W. H. Sebrell and R. D. Lillie *Proc Soc Exptl Biol Med* 48:228 (1941).

¹² P. György and H. Goldblatt *Proc Soc Exptl Biol Med* 46:492 (1941); P. György, E. C. Poling and H. Goldblatt *Ibid* 47:41 (1941).

¹³ I. L. Chalkoff and C. L. Connor *Ibid* 43:638 (1940).

¹⁴ A. R. Rich and J. D. Hamilton *Bull Johns Hopkins Hosp* 66:180 (1940).

¹⁵ M. A. Spellberg and R. W. Keeton *Am J Med Sci* 200:688 (1940).

¹⁶ W. H. Griffith and N. J. Wade *J Biol Chem* 131:567 (1939); 132:677 (1940).

¹⁷ W. H. Griffith *J Nutrition* 21:291 (1941); W. H. Griffith and D. J. Mulford *J Am Chem Soc* 63:929 (1941).

¹⁸ P. György and R. E. Bekhardt *Biochem J* 34:1143 (1940).

¹⁹ L. Rane and Y. S. Sbarrow *J Biol Chem* 134:455 (1940).

lymph nodes to hemolymph nodes, and in more severe cases hemorrhages in the eye have been observed^{10, 11} These compounds are also necessary for normal growth and lactation of the rat In the nursing young rat a flaccid paralysis of the hind leg occurs^{12, 22} In albino rats a rustiness of the fur has been observed as the result of a diet low in choline³

In chicks choline deficiency causes decreased or discontinued egg production, increased mortality and abortion of egg yolks⁴ In turkeys and in chicks choline deficiency is characterized by perosis and by slow growth^{25, 26, 27}

Choline deficiency is also manifested by high non protein nitrogen in the blood²⁸ Prolonged deficiency causes a loss of the ability of the liver to store glycogen and to excrete dye²⁹

4 Specificity Studies

The naturally occurring suppliers of the transferable methyl group show a relatively high specificity Many proteins are active due to their content of methionine or due to the fact that betaine may be formed from some of the amino acids in the protein³⁰ However the methyl groups of all compounds, which have methyl groups attached to quaternary nitrogen or sulfur are not transferable In order to be available for transmethylation, the methyl group must be bound on specific molecules Thus in choline the hydroxyl group must be free or in a readily available form since the ethers are inactive³¹ Choline salts such as choline chloride and betaine aldehyde are active³¹ On the other hand, the nitrogen of choline may be replaced by phosphorus³¹ without loss of activity The methyl groups of creatine,³² S methyl cysteine^{32, 34} or of the betaines from threonine, serine or allothreonine³ are not transferable

* K Christensen *Proc Am Soc Biol Chem* 1940 XX

¹¹ R W Engel and W D Salmon *J Nutrition* 22 109 (1941)

¹² B Sure *Ibid* 19 71 (1940)

¹³ H S Owens M Trautman and F Woods *Science* 93 406 (1941)

¹⁴ O D Abbott and C U DeMatters *J Nutrition* 19 47 (1940)

¹⁵ T H Jukes *J Biol Chem* 134 789 (1940) *J Nutrition* 20 445 (1940)

¹⁶ D M Hegsted R C Mills C A Elvehjem and F B Hart *J Biol Chem* 138 459 (1941)

¹⁷ T H Jukes *Proc Soc Exptl Biol Med* 46 155 (1941)

¹⁸ W H Griffith and D J Mulford *J Nutrition* 21 633 (1941) R W Engel and W D Salmon *Ibid* 22 109 (1941)

¹⁹ D L MacLean J H Ridout and C H Best *Brit J Exptl Path* 18 345 (1937)

²⁰ A W Beeson H J Channon J V Loach and H Wilkinson *Biochem J* 30 1040 (1936) C H Best R Grant and J H Ridout *J Physiol* 86 337 (1936)

²¹ A D Welch and M S Welch *Proc Soc Exptl Biol Med* 39 7 (1938)

²² V du Vigneaud J P Chandler M Cohn and G B Brown *J Biol Chem* 134 787 (1940)

²³ H J Channon M C Manfold and A P Platt *Biochem J* 34 866 (1940)

²⁴ A D Welch *J Biol Chem* 137 173 (1940)

²⁵ H E Carter and D B Melville *Ibid* 133 109 (1940)

Choline derivatives which contain alkyl groups other than the methyl group and even the methyl diethyl homolog³⁴ are unable to support growth of rats on a diet containing homocysteine,³⁶ but are potent lipotropic agents³⁷ and prevent the occurrence of hemorrhagic kidneys.³⁴ Arsenocholine, which is active as a lipotropic factor³¹⁻³⁵ and prevents the hemorrhagic kidney condition in rats and perosis in turkeys,³⁴ does not transfer the methyl group to homocysteine.³⁴

5 The Physiological Action of the Active Compounds

According to the tentative classification of choline as a vitagen, this substance is utilized in the animal organism as a building unit and is at the same time concerned with the regulation of metabolic processes. Choline serves as a building unit in many phosphatides for example, in lecithins and in sphingomyelins. Choline is present in the phospholipides of practically all cells. In addition, choline regulates the turnover of fat³⁸ and of phospholipides,⁴⁰ for example, in the liver and has for that reason often been classified as a vitamin.⁴¹ In the absence of choline, fat is deposited in the liver, but this deposition is removed when choline becomes available. A dietary deficiency of choline becomes apparent from disturbances of the fat metabolism in spite of the presence in the body of very large amounts of choline containing phospholipides.⁴ In addition to these functions, choline is utilized in the animal organism after acetylation as a blood pressure lowering substance. The process of producing acetylcholine is intimately connected with nerve impulses and special enzyme systems are readily available in the organism to acetylate choline when needed and to hydrolyze the active acetyl compound to the free choline which is relatively incapable of influencing the blood pressure.

Choline can be synthesized to a limited extent in the animal organism. This is apparent, for example, from the fact that deuterium containing choline can be isolated after feeding methionine the sulfur bound methyl group of which contained deuterium.⁴² Choline synthesis is furthermore indicated by the fact that the choline content of rats on choline-free diets

¹ V du Vigneaud, J. P. Chandler, A. W. Moyer and D. M. Keppel, *J. Biol. Chem.* 131: 57 (1939).

² H. J. Channon and J. A. B. Smith, *Biochem. J.* 30: 110 (1936). H. J. Channon, A. P. Platt and J. A. B. Smith, *Ibid.* 31: 1738 (1936).

³ A. D. Welch, *Proc. Soc. Exptl. Biol. Med.* 35: 107 (1936). C. H. Best and J. H. Ridout, *Can. Med. Assoc. J.* 39: 188 (1938).

⁴ See the review by C. H. Best and J. H. Ridout, *Ann. Rev. Biochem.* 8: 349 (1939).

⁵ J. Perlman and I. I. Chaikoff, *J. Biol. Chem.* 127: 211 (1938); 128: 735 (1939); 130: 593 (1939).

⁶ P. György and H. Goldblatt, *J. Exptl. Med.* 72: 1 (1940). C. G. King, *Ann. Rev. Biochem.* 8:

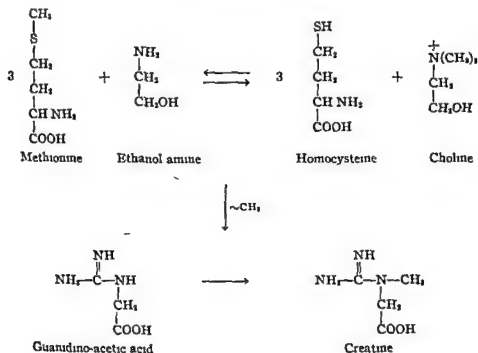
371 (1939). H. C. Sherman, *Chemistry of Food and Nutrition*, New York, 1941, p. 395.

⁷ F. X. Aylward, H. J. Channon and H. Wilkinson, *Biochem. J.* 29: 169 (1935).

⁸ V. du Vigneaud, J. P. Chandler, M. Cohn and G. B. Brown, *J. Biol. Chem.* 134: 787 (1940).

increased with the weight of the animals ⁴⁴ Preformed choline must, however be supplied to the animal for optimal physiological performance

The methyl groups attached to the quaternary nitrogen are partly responsible for the physiological action of choline They serve as methylating agents in anabolic processes for example in the synthesis of creatine from guanidino acetic acid Choline can be replaced in this reaction by other compounds with transferable methyl groups ($\sim\text{CH}_3$) such as methionine and betaine Another physiologically important methylation is the synthesis of methionine from homocysteine and choline On the other hand choline can be built up from methionine and ethanol amine This series of reactions is shown in the following scheme



The action of choline however, cannot be due solely to the available methyl groups For example, the ethyl derivatives of choline cannot transfer methyl groups since the compounds are devoid of them and either do not transfer the ethyl group or the ethyl group is inactive In any event, these compounds are unable to support growth of rats on a choline and methionine free diet in the presence of homocysteine ⁴⁵ Arsenocholine

H P Jacobi C A Bumann and W J Meek *J Biol Chem* 138 571 (1941)

⁴⁵ du Vigneaud J P Chandler A W Moyer and D M Keppel *Ibid* 131 57 (1939)

also is unable to transfer methyl groups⁴⁶ However, these compounds are active as lipotropic factors, and they prevent the hemorrhagic kidney condition and the perosis encountered in animals kept on a diet devoid of choline

6 Requirements

Choline (methionine or betaine) is required by all investigated animals, such as the rat, dog, rabbit, guinea pig, chick, turkey, etc. Choline is also an essential growth factor for bacteria, for example, for the *Pneumococcus bacillus*⁴⁷

Generally the young, growing organism needs more choline than the adult organism. During lactation the requirements are increased. Rats need 10-20 mg choline per day to prevent the deposition of excess amounts of fats in the liver and to maintain the fat deposition at a normal level.⁴⁸ The choline requirement of dogs is about 35 mg per kilogram of body weight.⁴⁹ Chicks need about 75 mg daily.⁵⁰

In the absence of choline, the animal organism is able to utilize effectively methionine or betaine. The efficacy of these compounds is, however, somewhat lower than that of choline (estimated efficacy 30%)

⁴⁶ A. D. Welch *Ibid.* 137 173 (1940)

⁴⁷ L. Rane and Y. Subbarow *Ibid.* 134 455 (1940)

⁴⁸ H. J. Chaunou, J. V. Loach and G. R. Tristram *Biochem. J.* 32 1377 (1938)

⁴⁹ C. Fritzman and I. I. Chaikoff *J. Biol. Chem.* 138 477 (1941)

⁵⁰ O. D. Abbott and C. U. DeMasters *J. Nutrition* 19 47 (1940)

ESSENTIAL ORGANIC SULFUR-CONTAINING COMPOUNDS

There is the possibility that organic chemical compounds containing sulfur belong to the class of protective foods¹ and should be classified as vitamins. Sulfur containing organic compounds act not only as structural building units for example in hair and nails, but are also of specific and functional importance for growth and maintenance of life. A deficiency of sulfhydryl compounds brings about death. Sulfhydryl compounds apparently act as activators (and inhibitors) of a number of enzyme systems probably by virtue of their ability to undergo reversible oxidation to disulfide compounds and reduction to the original sulfhydryl compounds. They act, for example, as activators in the aerobic and anaerobic fermentation and in the oxidation of glucose in propionic acid bacteria.² In experiments with the *Amoeba proteus* it has been found³ that sulfhydryl compounds regulate nuclear growth and fission and in experiments with non nucleated blue green algae, a stimulating influence on cell multiplication has been observed.⁴

The demonstration of the physiological significance of sulfhydryl compounds is however, in itself, no proof for their vitamin nature. This rests with the demonstration that the organism is unable to synthesize a particular sulfur containing compound which may be essential, such as cysteine or the cysteine containing glutathione or any other normal body constituent of this class. There is actually ample experimental evidence that cysteine is synthesized in the organism^{5,7} from methionine.⁸ On the other hand the essential nature of methionine has been established in the search for the indispensable amino acids (see page 540) and its efficacy is at least partly due to the available methyl group which acts in transmethylation (see page 543). Whether or not the vitamin action of methionine involves a participation of the sulfur in the molecule is not known. Future research must decide whether or not sulfur or sulfhydryl compounds belong to the class of vitamins and if the activity is due to the presence of the sulfur group, sulfhydryl group or due to the entire molecule.

Among the known vitamins is a sulfur containing compound namely,

¹ W. C. Rose *Science* 86 298 (1937) W. H. Griffith *J. Nutrition* 21 991 (1941)

² See the review by T. Bersin *Ergeb. Enzymforsch.* 4 68 (1935)

³ P. Chaux and C. Fromageot *Enzymolog.* 6 33 (1939)

⁴ C. Voegtlin and H. W. Chalkley *U. S. Pub. Health Service Pub. Health Repts.* 45 3041 (1930) *J. Natl. Cancer Inst.* 1 63 (1940)

⁵ F. S. Hammett and L. Walp *Growth* 3 477 (1940)

⁶ W. C. Rose *Physiol. Rev.* 18 109 (1938)

⁷ E. F. Bech and A. White *J. Biol. Chem.* 127 87 (1939)

⁸ H. Tarver and C. L. A. Schmidt *ibid.* 130 67 (1939)

vitamin B₁ While vitamin B₁ is not a sulfhydryl compound but a sulfide, the mechanism of the vitamin action is believed to be due to the ability of the vitamin to undergo reversible oxidation reactions with the intermediary formation of a disulfide (see page 143) It is also known that in this case the vitamin action is due to the specific structure of the entire molecule

PATENT INDEX

PATENT INDEX

In the following pages United States British German and French patents dealing with vitamins are listed They are divided according to subject matter and arranged numerically under each subject The essential data of each patent are given including the number of the patent and the country in which it issued (U S for United States for Great Britain G for Germany and F for France) This is followed by the date at which the patent was issued The inventor and the assignment recorded at the date of issue are indicated A short abstract of the claim of the patent follows

In addition to patents from the United States Great Britain Germany and France a few patents from other countries such as Austria Australia (Austral) Belgium (Belg) Canada (Can) Denmark (Den) Japan (Jap) Norway (Norw) Russia (Russ) Sweden (Swed) and Switzerland (Swiss) are mentioned because of their outstanding interest

Abbreviations have been used for a number of industrial organizations

Ciba stands for Chemical Industries of Basel
 DuPont stands for E I du Pont de Nemours & Co
 Glaxo stands for Glaxo Laboratories Ltd
 Hilger stands for Adam Hilger Ltd
 I C I stands for Imperial Chemical Industries
 I G stands for Interessen Gemeinschaft der Farben
 industrie
 Pfizer stands for Charles Pfizer & Co Inc
 Squibb stands for E R Squibb & Son

VITAMINS GENERAL

PATENT NO	DATE	PATENTEE	ABSTRACT
S 883 174	Mar 31 1908	F S Davidson and W P Burra	Extracts from hops and yeasts by steaming under pressure followed by pressing to separate the condensed liquid extract
S 1461703	July 10 1923	D Ch dlow	Preserving the vitamin content of cereals by heating to 132 C
S 1474746	Nov 20 1923	G S Ward	Bread enriched with all vitamins of natural origin
S 1479418	Jan 1 1924	N Min nberg	Separation of vitamins of whole grain cooking of the starchy extracted material addition of the vitamin extract to the cooked extracted material and drying
S 1479502	Jan 1 1924	G Heffele	Yeast mixture as vitamin food
S 1480520	Jan 8 1924	J R Eoff	Vitamin extract from yeast or yeast concentrate
S 1481671	Jan 22 1924	T J Allen	Vitamin-containing food by fermentation of yeast

PATENT NO	DATE	PATENTEE	ABSTRACT
U S 1 488 815	April 1 1924	I F Harris	Vitamin extract from yeast
U S 1 502 563	July '2 1924	W P Heath	Preserving vitamins in bread by working in an atmosphere of CO ₂
U S 1 526 032	Feb 10 1925	J A Wessener	Vitamin-containing food by mixing steep water with yeast followed by evaporation
U S 1 538 366	May 19 1925	R Willstätter and H Sobotka	Yeast rich in vitamins
U S 1 540 883	June 9 1925	I F Harris	Vitamin preparations especially water soluble vitamins are preserved from deterioration by admixture with non hygroscopic sugars
U S 1 541 263	June 9 1925	C Hoffman H D Grigsby and N M Cregor	Vitamin containing food ingredients from rice polishings wheat bran or cereal germ by cooking or by proteolytic enzymatic action followed by saccharification by malt diastase
U S 1 552 176	Sept 1 1925	H Liebers	Production of nutrient materials rich in vitamins by mixing yeast with concentrated extracts of germinated cereals.
B 242 645	Nov 8 1925		
U S 1 552 549	Sept 8 1925	B L Eicher	Charcoal tablets containing medicinal and fish oils
B 258 704	Oct 6 1926		
F 611 760	Oct 11 1926		
U S 1 568 196	Jan 5 1926	O Stiner A Hauswirth and A Gams	Vitamin malt preparation
U S 1 574 776	Mar 2 1926	R Willstätter and H Sobotka	Yeast preparation rich in vitamins
U S 1 589 192	June 15 1926	T C Manchester	Preservation of the vitamin content of milk by replacing the air in receptacles containing the milk with an inert gas such as CO ₂
U S 1 590 837	June 29 1926	H Liebers	Addition of yeast to cheese to increase its vitamin content.
U S 1 624 154	April 12 1927	M Winckel	Vitamin preparation from fermented skim milk and yeast.
U S 1 633 711	June 28 1927	R. K. Prince Ass to Vitamin Food Co	Process for sealing vitamins A and D from cod liver oil into a mixture of yeast or other vegetable products with a gum solution
U S 1 690 091	Oct. 30 1928	J K. Marcus	Separation of vitamins A, D and E along with unsaponifiable matter from oils by saponification and extraction with ethylene dichloride
B 289 798	April 30 1927		
G 545 268	May 1 1928		
F 655 799	April 27 1928		
U S 1 708 914	April 9 1929	B Dass Ass to Ellis Foster Co	Incorporation of dried yeast into peanut butter
U S 1 722 175	July 23 1929	W S. Bowen	Spray desiccation for material containing vitamins
U S 1 746 657	Feb 11 1930	W J Kemp	Preparation of vitamin-containing tomato juice
U S 1 753 531	April 8 1930	R. K. Prince Ass to Vitamin Food Co	Endocrine gland substances are mixed with vitamin preparations.
U S 1 756 574	April 29 1930	J Takamine J Takamine and N Fujita Ass to Takamine Ferment Co	Vitamin product from propagated fungi such as aspergillus

PATENT NO	DATE	PATENTER	ABSTRACT
U S 1764 085	June 17 1930	H Placek Ass to G R Conkey Co	Vitamin containing granular products from cod liver oil yeast or soy bean meal and paraffin
U S 1775 966	Sept 16 1930	E H Miles and G Reilly	Preparation of vegetable extracts rich in vitamins by mixing the juices of vegetables containing vitamins and preservatives adding acid material and heating the mixture to accomplish hydrolysis
U S Reissue 18 542	July 26 1932		
U S 1775 967	Sept. 16 1930		
U S Reissue 18 523	July 12 1932		
B 236 785	Aug 19 19 6		
B 274 051	July 8 1920		
U S 1796 077	Mar 10 1931	H Iscovesco Ass to Health Products	Process of isolating lipoids by acetone extraction
U S 1845 813	Feb 16 1932	R K Prince	Vitamin concentrate from a blend of different vegetables
U S 1918 983	July 18 1933	Ass to Vi Foods Co	
U S 1861 677	June 7 1932	L A Agopian	Vitamin concentrate from fruits or vegetables by precipitation with a Pb- or Cu salt followed by extraction of the precipitate
B 268 655	Sept 24 1926		
G 486 228	Sept 25 1926		
U S 1879 762	Sept 27 1932	F W Nitardy Ass to Squibb	Vitamin-containing tablets are provided with a coating containing an antioxidant such as hydroquinone
U S 1886 931	Nov 8 1932	E R Alexander Ass to Vitamin Co of America	Mixing of vitamins with citrus fruit masses
U S 1896 490	Feb 7 1933	E Komm	Vitamin-containing food product from wheat germ
U S 1896 500	Feb 7 1933		
U S 1896 621	Feb 7 1933		
U S 1913 518	June 1 1933	C Schmitt	Vitamin extract from cotton seeds
B 317 554	May 25 1928		
F 655 181	June 2 1928		
U S 1919 297	July 25 1933	W Kropp F Lange and A Bohne	Stable aqueous solutions of fat soluble vitamins by using water soluble amides of lower fatty acids as solvents for the vitamins
G 508 503	Oct 23 1928	Ass to Winthrop	
U S 1929 786	Oct 10 1933	A E Meyer Ass to Chappel Brothers	Isolation of chondroitin compounds from cartilage
U S 1942 943	Jan. 9 1934	C F Schnabel	Poultry feed rich in vitamins from grass
U S 1964 867	July 3 1934	L B Allyn Ass to Vitamin Food Co	Granules of dried yeast impregnated with a vitamin A-containing oil and paraffin
Continuation of U S 1833 711			
U S 1965 353	July 3 1934	C L Patterson	Vitamin-containing foodstuff from milk and yeast
U S 1975 169	Oct 2 1934	A B O Norrbom	Extraction of water soluble vitamins by dilute acids and addition of a substance soluble in acid solution which precipitates upon neutralization and carries the vitamins in the precipitate
U S 1984 853	Dec 18 1934		
B 441 313	Jan 21 1936	C L Barthen	Countercurrent extraction of vitamins from an aqueous alkaline soap
F 782 314	June 3 1935	Ass to Health Products Co	
U S 1988 677	Jan 22 1935	G D Arnold	Portable apparatus for drying vegetable matter while preserving the vitamin content
U S 1988 678	Jan. 22 1935		

PATENT NO	DATE	PATENTEE	ABSTRACT
U S 1 999 789	April 30 1935	E Schmierer Ass to Baker Perkins Co	Apparatus for extracting vitamins containing juices from fruits and vegetables in a vacuum
U S 2 006 700	July 2 1935	G C Supplee G E Flanagan and R C Bender Ass to The Borden Co	Method for separating vitamins from casein
U S 2 017 942	Oct 2 1935	S Botchinsky Ass to S Tittelbaum	Fat soluble vitamins are obtained from animal or vegetable material by plasmolysis followed by extraction
U S 2 041 056	May 11 1936	W L Plesher	Vitamin containing compounds are injected into freshly baked products such as bread
U S 2 041 179	May 19 1936	C Hoffman Ass to Ward Baking Co	Cereal food is enriched in vitamins by adding natural vitamin containing ingredients to dough
U S 2 052 218	Aug 25 1936	C Dickens	Vitamin product by concentrating milk
U S 2 052 219	Aug 25 1936	C Dickens	Vitamin concentrate from asparagus
U S 2 060 389	Nov 10 1936	A I Wigelsworth	Apparatus for drying organic materials while preserving their vitamin content
U S 2 063 332	Dec 1 1936	C W Kirby and C N Frey Ass to Standard Brands	Vitamin product from yeast
U S 2 072 402	Mar 1937	F Kretschmer Ass to Krause Medico	Vitamin containing food product from unripe apples
U S 2 128 845	Aug 30 1938	R P Myers and S M Weisberg Ass to Sealtest System Laboratories	Whey is fermented with a lactose fermenting microorganism to produce a product rich in vitamins
U S 2 178 946	Sept 6 1938	M B Katzman	Tetraphosphates of aliphatic hydroxy compounds are used to retard rancidification of vitamin containing preparations
U S 2 137 606	Oct 11 1938	A R Smith Ass to Combustion Engineering Co	Flash drying of materials containing vitamins
U S 2 133 367	Oct 18 1938	C F Schnabel Ass to American Dairies	Vitamin concentrate from grass juices
U S 2 141 40	Dec 27 1938	C Waizmann	Vitamin products from yeast and vegetable material
U S 2 140 344	Jun 31 1939	I Draibach Ass to Hall Laboratories	Vitamin in oil water emulsions using an alkali metaphosphate as the emulsifying agent
U S 2 151 644	Mar 1 1939	H C Stephens Ass to Natural Food Products	Deaeration of fruit juices to preserve the vitamin content
U S 2 157 750	May 9 1939	C G Harrel and A W Lindert Ass to Pillsbury Flour Mills Co	Addition of an oil soluble dye to vitamin preparations to facilitate the degree of mixing with other feed
U S 2 167 144	July 20 1939	R W Barton and W M Cox Ass to Mead Johnson	Vitamin emulsions containing glycerol a mono oleic acid ester of di glycerol and sucrose

PATENT NO	DATE	PATENTEE	ABSTRACT
U S 170 070	Aug 22 1939	J A Reynolds Ass to Atlantic Coast Fish eries Co	Gelatine capsules for vitamin bear ing liquids
U S 2 183 053	Dec 12 1939	H P Taylor	Incorporation of vitamins in gela tine droplets
B 489 970	Aug 2 1938	Ass to Atlantic Coast Fish eries Co	
B 490 001	Aug 2 1938		
U S 2 186 82	Jan 9 1940	W W Cowgill	Apparatus for drying vitamin con taining foods in film form
U S 2 133 319	Jan 30 1940	G F Siemens Ass to McKesson & Robbins and to Vital	Compo tion of vitamin A with an adsorbate of vitamins B ₁ and B
U S 2 189 438	Feb 6 1940	L H Smith and C F Schna bel Ass to American Dairies	Vitamin-containing fodder by mix ing dried greens with a milk prod uct
U S 2 191 6	Mar 26 1940	C M Porter L V Porter and E Hurlock	Food product rich in vitamins from lactobacillus cultures
U S 2 195 593	Apr 12 1940	F W Nitardy Ass to Squibb	Stabilization of fatty materials con taining vitamins by the addition of an anti acid substance such as MgO
U S 2 195 90	April 2 1940	F W Nitardy	
U S 2 08 113	July 2 1940	Ass to Squibb	Vitamin tablets containing Ca gluconate or Ca phosphate
U S 2 197 095	April 16 1940	B Cuenod	Addition of vitamins to milk prepa rations
B 4 8 399	Dec 18 1936	Ass to Soc d'etude et appli cations industrielles	
B 473 506	Oct 14 1937		
F 797 654	May 1 1936		
U S 2 200 319	July 2 1940	F Ge tz	Addition of vitamins to ground cof fee by a spray process.
U S 2 200 517	April 1 1941	F J Cahn and B R Harris Ass to Emul of Co	Emulsification of vitamins with a reaction product of an anhydride of a carboxylic acid ester of a hydroxy polycarboxylic acid and a partial ether or ester of an aliphatic poly hydroxy-compound
B 19,343	April 5 1923	F M Traiss and C Glaba	Vitamin-containing milk by addi tion of fish oils malt extracts and yeast
B 199 043	June 11 1923	P Farup	Addition of alcohol to fish oils Also addition of plant extracts
B 217 8	Mar 5 1923	Kellogg Co	Vitamin extracts from bread milk or other sources are added to dough or bread preparations
C 431 158	Sept 20 1928		
B 20 3	Sept 17 1923	J Schmidt	Vitamin-containing materials such as yeast are added to products similar to malt extracts
B 2 5	May 29 1923	Fleischmann Corp	Addition of vitamins to yeast nutri ent solutions
B 2 6549	Dec 21 1923	Mellemeuro Pae sk	Vitamin preparations are added to cheese
B 237,442	July 16 1924	O Mustad and Son	Margarine and other edible fatty materials are mixed with substances rich in vitamins in the presence of an inert gas
B 49 746	April 8 1926	P M W Greich	Vitamin-containing food from mixed grain
C 456 387	Feb 21 1928		
F 604 077	April 8 1926		

PATENT NO	DATE	PATENTEE	ABSTRACT
B 254 724	July 1 1925	F H Peck	Addition of vitamins to beverages. Also ultraviolet light irradiation of beverages
B 271 626	July 20 1927	H Hadden	Vitamin-containing tablets from dried vegetables or fruits
B 280 212	May 17 1928	S Grønningsæter and Fischer Hollinshed Co	Process of transferring vitamins by extracting the vitamins from vitamin-containing material and adding the substance to be vitaminized to the solution containing the extract
B 293 735	April 11 1927	Vitamin Food Co	Dried yeast and cod liver oil are mixed with a gum and dried to form an air excluding coating on the granular particles.
B 297,256	Dec. 14 1927	J E Nyrop	Production of vitamin-containing powders suitable for adding to margarine by spray-drying emulsions containing cream or skimmed milk
B 315 340	Jan 10 1928	W Kollath and H Magistris Ass to L. W Gans A G	Vitamin extract by precipitation and absorptions
B 320 369	July 2 1928	J Korselt	Vitamin solutions from plant juices are treated with an acid Ca salt to remove oxalic acid.
B 326 742	July 25 1929	Van Den Bergh s Margarine Ges	Incorporation of emulsions of vitamins into margarine.
G 487 494	June 18 1921		
B 328 942	Feb 4 1929	Matro Ges	Vitamins are extracted from root
B 362 073	Sept 11 1930		lets of grain from malt houses
F 655 343			
and Add			
39 331	Sept 8 1930		
B 340 580	Nov 24 1928	VanHouten & Zoon	Addition of vitamins to chocolate and cocoa
B 360 282	Jan 23 1931		
F 681 093	Sept 2 1929		
B 343 086	Nov 13 1929	A B O Norbin and A Astra	Extraction of water soluble vitamins by diluted acids precipitations and extractions
G 570 158	Nov 14 1929	Apotekarnas Kemiska Fabriker	
B 346 574	June 15 1929	U Brunch H Spehr and C E	Preservation of vitamins in dried fruits by the addition of acids and salts prior to the drying
G 519 778	June 16 1929	Sander	
F 696 910	April 15 1930	Ass to Brunch and Spehr	
B 750 684	May 14 1930	E Maybury	Food seasonings and sauces containing vitamins
B 757 732	Sept 13 1929	H Van de Sandt	Addition of vitamins to beer
G 598 027	Jan 3 1935		
G 609 744	Feb 22 1935		
G 618 899	Oct 31 1935		
G 625 075	Feb 3 1936		
G 636 434	Oct 9 1936		
F 698 375	July 4 1930		
B 362 023	Dec 9 1931	Matro Ges	Production of vitamin extracts
G 501 844	July 9 1930		
G 647 542	July 7 1937		
B 365 256	Dec 1 1930	H Van de Sandt	Dealbuminized vitamin concentrate from yeast tomatoes spinach etc.

PATENT No	DATE	PATENTEE	ABSTRACT
B 366 516	Oct 19 1929	L Bernardini	Fat and water soluble vitamins from germs and cereals are added to food products
B 367 063	Nov 29 1929	H Krönig	Enrichment of vitamins in beer by the addition of opened yeast
B 367 909	Dec 3 1929	Ass to F Lux	
B 368 919	Jan. 30 1930		
C 579 369	June 24 1933		
B 369 633	Dec 23 1930	W W Triggs	Vitamin products from citrus fruits liver oils wheat germ oils etc
F 709 423	Aug 6 1931	Ass to Tropical Vitamin Co	
B 370 926	April 6 1932	J E Nyrop	Vitamin a-containing feed for animals
B 378 399	Mar 24 1931	L W Mapson J T Mac Curdy H O Nolan and Cam bio Products Ltd	Edible vitamin-containing food products by autolysis or fermentation of animal or plant material
B 395 957	July 27 1933	A R Jahn	Multiple effect evaporator for the concentration of vitamins
B 418 214	Oct 8 1934	Salsterol Lab	Aqueous vitamin concentrate from raw fresh plant tissue
B 425 998	Mar 26 1935	I G	Emulsions of fat soluble vitamins in oil with water and an emulsifying agent such as gelatin gum arabic yolk of egg etc
B 454 528	Oct 2 1936	A Nyrop	Vitamin extract from pulped raw material by treatment with steam under pressure
B 459 467	Jan 8 1937	Nyegaard & Co	Vitamins are incorporated in CO ₂ evolving compounds containing substances of acid reaction
B 506 09	May 23 1939	I G	Manufacture of edible glycerides to which vitamins may be added
G 362 367	Oct 7 1922	T Hamburger	Dry vitamin preparation from plant juices and calcium lactate
G 39 442	Mar 21 1924	Bayer	Food product from playmolized yeast and calcium phosphate
G 470 035	Jan 3 1929	M Winkel	Vitamin containing yoghurt
G 483 394	Aug 12 1925	H Aman	Extract of vitamins and inositol phosphoric acid
G 489 186	May 13 1926	O Reinke	Vitamin extract from asparagus
G 49 281	Feb 20 1930	J Wolf	Extraction of vitamins from fish livers by aprotic solvents
G 499 384	Aug 1 1925	Aktienfabrik zur Erzeugung von Chemikalien	In the preparation of vitamin extracts the starting material is playmolized followed by saccharification
G 504 816	June 7 1922	Dimitt A G	Vitamin products from yeast
G 505,356	Aug 18 1930	P Grube	Vitamin-containing food product from the berries of <i>Salsola vermiculata</i>
G 511 993	Mar 28 1929	H Jena and J Jena	Addition of vitamin-containing fruit juices to cheese
G 518 820	July 29 1927	R Neugebauer	Food rich in vitamin from germinating cereals
G 521 16	Aug 29 1926	Knoll A G	Purification of vitamins by fractionation and distillation

PATENT NO	DATE	PATENTEE	ABSTRACT
G 528 200	Feb 12 1930	A Keddi	A vitamin extract from barley to be added to dough
G 532 521	Mar 20 1927	H Netzw & Co	Vitamin preparations from irradiated yeast are added to margarine
G 537 057	May 19 1928	Enosis S A	An aqueous vitamin extract from cotton seeds is added to milk and milk products
G 549 304	Feb 26 1930	H Van de Sandt	Stabilization of beverages to which vitamins have been added by adjusting the pH to about 4
G 561 686	April 1 st 1927	Vitamin Food Co	Bird food rich in vitamins from yeast cod liver oil and gum
G 565 901	April 2 nd 1931	A Hölscher	Vitamin product by fermenting vegetable material containing vitamins with yeast
G 57 546	April 13 1933	H Van de Sandt	Vitamin containing beer
G 577 622	May 11 1933		
G 631 18	June 10 1936		
G 581 143	July 21 1933	H Kronig	Beer is enriched in vitamin content by adding disintegrated yeast before the end of the fermentation
Add to G 561 720			
G 586 589	Oct 25 1933	F Weinmann	d Glucuronic acid from plant germs by hydrolysis
G 586 909	Oct 27 1933	W Jesselberg	Vitamin preparations are mixed with albuminous material and dried
G 618 482	Sept 9 1930	H Sander & Co	Food preparations containing extracts of water and fat soluble vitamins
G 623 610	Dec 30 1930	K Bodeendorf	Food preparation by extracting vegetable matter with cod liver oil
G 623 657	Jan 2 1936	W Kropp F Lange and A Bohne Ass to I G	Preparation of water emulsions of fat soluble vitamins by dissolving the vitamins in water soluble ethers or esters of polyhydroxy-compounds followed by incorporation into water
G 626 776	Mar 2 1936	Hoffmann LaRoche	Extraction of vitamins with solutions of bile acids followed by separation of the acids
G 642 307	Mar 1 1937	F Laquer Ass to I G	Dispersions of fat soluble vitamins in water by means of albuminous materials
G 661 503	June 20 1938	P Lindner	Assimilation of alcohol and/or CO ₂ by yeast or algae to form vitamins
F 694 443	July 24 1939	L M Rayband	Preservation of vitamins in germinated seeds by a coating of sugar chocolate
F 715 445	Aug 26 1930	A A Gournier	Food product rich in vitamins from cereals and milk
F 717 067	May 15 1931	I G	Dispersion of vitamins in water by the aid of albumins
F 723 537	July 18 1931	G Dubois	Extraction and concentration of vitamins
F 735 596	April 20 1933	Établissements Byla	Food products from yeast

PATENT NO	DATE	PATENTS	ABSTRACT
F 757 454	Dec 27 1933	H C Meyers	Food product rich in vitamins from the germs of grain
F 771 059	Sept 29 1934	M C Gulbraansen	Concentrated vegetable juices are mixed with vitamin containing liquids
F 796 101	Mar 30 1936	M Ernotte	Vitamin composition containing lecithin sugar and vitamins especially vitamin D
F 797 180	April 29 1936	E A Harbet	Vitamin enriched drinks by the addition of concentrates from grapes
F 797 229	April 23 1936	International Vitamine Lab Co	Fresh foods are dried to preserve their vitamin content by a liquid which causes exosmosis of the juice
F 805 598	Nov 4 1936	Soc Française Des Sucres	Addition of vitamins to sugar
F 810 961	April 3 1937	C G V Bouillon	Apparatus for generating vitamins in grain
F 833 984	Nov 8 1938	Ocean	The vitamins in a purée of paprika are preserved by sealing under vacuum
F 845 046	Aug 9 1939	G Sandulesco and A Girard Ass to Les Laboratoires Français de Chimiothérapie	Separation of hydroxyl group containing vitamins by transformation into water soluble quaternary ammonium compounds
Austral 9 933/35	Jan 16 1936	Nestlé and Anglo-Swiss Condensed Milk Co	Fat and water soluble vitamins are emulsified in condensed milk
Austrian 121 078	Aug 15 1930	E Reinisch	Yeast is enriched in vitamins by ultraviolet rays
Austrian 131 289	July 15 1932	H Oleoth	Extraction of vitamins from vegetable materials or microorganisms in the presence of nascent hydrogen
Austrian 137 47	May 11 1934	M Klein	Vitamin preparations from cellular vegetable tissue by dialysis followed by precipitation and extraction procedures
Den 54 611	April 19 1938	H C E Tjisch	Vitamin-containing material for coloring margarine is extracted from plants with oils
Norw 43 89	Mar 28 1927	A W Owe	Vitamin extract from vegetable material by saponification and extraction
Norw 44 019	Dec 29 1930	A W Owe	Edible fat is mixed with a vitamin bearing material
Vitamins A and D			
U S 1 162 907	Dec 7 1915	C Funk	Extraction of vitamins from cod liver oil with ligroin or another organic solvent precipitation with alcohol or acetone
U S 1 3 6968	Jan 6 1910	G D Roger	Oils separated from fatty materials e.g. from fish livers by adding sodium chloride followed by subjecting to an electrical discharge
U S 1 368 148	Feb 8 1911	P M Heyrdahl	Fractionation and purification of oils e.g. fish oils by water
U S 1 519 779	Dec 16 1924	C M Johnson	Oil is pressed from frozen cod livers.

PATENT NO	DATE	PATENTER	ABSTRACT
Vitamins A and D (Continued)			
U S 1 629 074	May 17 1927	C Funk and H E Dubin	Vitamins A and D are obtained from fish oils by extraction with acetic acid saponification and digitation precipitation of non vitamin products
U S 1 629 618	May 24 1927	S Grönningaer	Vitamin containing fats are saponified with alcoholic alkali and the vitamins extracted by a vegetable oil
U S 1 638 700	Aug 9 1927	D Molofsky Ass to Silmo Chemical Co	Powdered fish oil product in a non metallic mineral carrier
U S 1 649 520	Nov 15 1927	C Funk and H E Dubin	Stabilizing vitamin containing oils by mild hydrogenation
U S 1 678 454	July 24 1928	T F Zucker	Extraction of liver oils with alcohol
B 208 145	April 3 1925	Ass to University Patents Corp	saponification of the extract precipitation of the fatty acids as calcium soaps and extraction of the vitamins from the soaps
B 227 121	April 3 1925		
B 227 122	April 3 1925		
G 484 993	Oct 24 1929		
F 579 734	Oct 22 1924		
U S 1 715 945	June 4 1929	A W Owe	Vitamin containing oils are saponified with an alkaline earth hydroxide and extracted with an edible fat
G 472 814	Oct 11 1924		
U S 1 725 964	Aug 27 1929	F W Nitardy Ass to Squibb	Vitamin bearing oils from fish livers by heating under subatmospheric pressure
U S 1 753 790	April 8 1930	K Kawai	Partial saponification of cod liver oil
U S 1 786 090	Dec 23 1930	K. Takahashi	Extraction of fat soluble vitamins from fish oils by saponification and precipitation of the fatty acids with an alkaline earth metal followed by extraction of the vitamins with an organic solvent
B 220 697	Aug 14 1924	Asst to Z H R Kenkyujo	
F 566 695	Feb 18 1924	Japan	
U S 1 805 593	May 19 1931	A W Owe	Vitamin extracts from marine oils by saponification followed by extraction with a vegetable oil
B 266 900	Mar 2 1926		
G 501 834	Nov 4 1924		
U S 1 845 370	Feb 16 1932	T B Wagner	Emulsions of cod liver oil with a calcium phosphorus compound derived from steepwater of corn
U S 1 879 734	Sept 27 1932	W G Christiansen and E Moness Ass to Squibb	Extraction of vitamins from a saponified material by means of acetone
U S 1 896 185	Feb 7 1933	H O Nolan Ass to Ellis Foster Co	Partially hydrogenated cod liver oil containing its original vitamin content
U S 1 897 039	Feb 14 1933	W G Christiansen W S Jones and E Moness Ass to Squibb	Precipitation of fatty acids in the saponification mass of cod liver oil as the Al salt
U S 1 919 369	July 25 1933	H A Holaday and A Black Ass to Squibb	Saponification of vitamin containing oils and extraction with ether acetone or dichloro ethyl ether
U S 1 925 489	Sept 5 1933	E Langfeldt and R Hellerud	Apparatus for the extraction of vitamins from solutions containing water soluble soaps by means of a vaporized solvent

PATENT NO	DATE	PATENTER	ABSTRACT
Vitamins A and D (Continued)			
U S 1935 042	Nov 14 1933	A Black Ass to Squibb	Refining the vitamin-containing unsaponifiable part of fish oils by dissolving in alcohol or dioxane and washing with a vegetable oil
U S 1947 315	Feb 13 1934	W O Snelling	Vitamin concentrate from fish oils by saponification in pentane with aqueous alkalis.
U S 1947 432	Feb 13 1934	R C Huston and H D Lightbody Ass to State Board of Agriculture of Michigan	Preservation of vitamin-containing fish liver oils by the addition of hydroquinone resorcinol or the like
U S 1983 654	Dec 11 1934	A Black Ass to Squibb	Refining the vitamin-containing unsaponifiable matter of oils by treatment with carbon in the presence of an antioxidant
U S 1988 969	Jan 22 1935	A F O Germann Ass to S M A Corp	Vitamin A and D concentrate from palm oil by separation of the fraction permanently liquid at low temperature and irradiation or addition of viosterol
U S 2007 108 B 463 655	July 2 1935 April 5 1937	H Bresnick	Vitamin preparation by mixing an edible dehydrated hard vegetable fat with a fatty material of high vitamin content
U S 2076 395	Dec 31 1935	H P Loomis Ass to Sulmo Chemical Co	Vitamin concentrate from oils by partial saponification
U S 2051,257	Aug 18 1936	H N Holmes Ass to Parke Davis	Materials such as fish liver oil carotene or irradiated ergosterol are stabilized by adding phospholipides such as lecithin sphingomyelin etc
U S 2067 279	Jan 12 1937	F W Nitardy and W S Jones Ass to Squibb	Vitamin-containing oil is extracted from fish livers the protein of which has been coagulated by means of ethylene chloride or other chlorinated solvents.
U S 2090 738	Aug 24 1937	A O Tischer Ass to Eastman Kodak	Purification of vitamin A and D containing natural oils by extraction of the impurities with an aldehyde having a furane nucleus
U S 2136 453	Nov 15 1938	H M Merker Ass to Parke Davis	Purification of vitamin-containing ether extracts from fish oils by means of sodium aluminum silicate.
U S 2136 481	Nov 15 1938	F H Young and H D Robinson Ass to Abbott	Vitamins A and D concentrate by partial saponification of livers
U S 2180 315 B 606 730	Mar 14 1939 June 5 1939	A E Briod and B R East Ass to National Oil Products	Process for emulsifying vitamins A and D concentrates with cream or evaporated milk
U S 2161 882	June 13 1939	James A. Patch	Method of extracting vitamins from fish liver oils by saponifying adding organic solvents to produce a homogeneous solution and subsequently adding excess water
U S 2173 629	Sept 19 1939	N A Miles Ass. to Research Corp	Process for the isolation of substantially pure vitamins A and D from fish liver oils by a series of fractional crystallizations

PATENT NO	DATE	PATENTEE	ABSTRACT
Vitamins A and D (Continued)			
U S 2 179 917	Nov 14 1939	H Brinton	Addition of iodine to vitamin A and D containing oils as a stimulating effect for poultry
U S 2 201 061 2 201 062 2 201 063 2 201 064	May 14 1940	B H Thurman Ass to Refining Inc	Stabilization of fats and oils by a vegetable phosphatide
U S 2 207 712	July 16 1940	J G Blaso Ass to Natural Vitamins Co	Concentration of vitamins in oils by hydrogenation of the oil removal of the hardened material and recovery of the vitamin containing mother liquors
U S 2 255 875	Sept 16 1941	L O Buxton and F J Simons	Purification of fat soluble vitamin containing oils by treatment with activated carbon
B 635 014	Mar 26 1941	Ass to National Oil Products	
U S 2 258 671	Oct 14 1941	L O Buxton Ass to National Oil Products	Refinement of vitamin containing oils by treatment with sugars
U S 2 258 672	Oct 14 1941	L O Buxton and H B Colman Ass to National Oil Products	Process for refining vitamin containing oils by treatment with aliphatic aldehydes
U S 2 258 673	Oct 14 1941	I O Buxton Ass to National Oil Products	Process for refining vitamin containing oils by treatment with dextrin
U S 2 266 719	Dec 16 1941	L O Buxton and F J Simons Ass to National Oil Products	Process for refining a fat soluble vitamin containing material with an adsorbent in a non polar solvent containing about 10% of a polar solvent
U S 2 266 830	Dec 23 1941	H F Taylor A W Wells and V A Nedzvedsky Ass to Atlantic Coast Fisheries	Vitamin concentrate from oils by saponification followed by extraction
B 301,000	Feb 10 1910	Boehringer	Extraction of vitamins from cod liver oil with ligroin and precipitation with phosphotungstic acid and extraction with acetone
B 207 545	Dec 5 1923	P M Heyerdahl	Liver oils are added to food products e g to chocolate or plant juices
B 214 238	April 9 1923	F W Nitardy Ass to Squibb	Packing of cod liver oil and similar food substances in the presence of an inert gas
B 217 363	April 9 1923	T W F Clark and F F Pearson & Co	Improvement in odor and flavor of fish oils or their unsaponifiable part by passing a stream of inert gas through the oil followed by heating
B 227 474	Jan 11 1924	D A Hausen	Oils are obtained from fish livers by passing inert gas through the material at 40-50 C
B 266 139	Mar 2 1926	A W Owe	Vitamin concentrates are added to oils and the mixture subjected to a mild refining treatment
B 267 410	Mar 17 1927	H Iscovesco and A B Adams	Extraction of vitamins from cod livers by saponification followed by extraction of the vitamins with a solvent

PATENT NO	DATE	PATENTEE	ABSTRACT
Vitamins A and D (Continued)			
B 289 187	Feb 7 1927	F H Carr Ass to British Drug Houses	Extraction of livers with an oil
B 293 777	April 5 1927	L H Lampitt and J H Bushill Ass to J Lyons & Co	Fats of high vitamin content from frozen viscera by heating with alkali solution and separating the fat
B 300 929	Feb 11 1928	K. Helholt	Vitamin containing oils are added to non saponifiable oils saponified extracted with a solvent and added to another oil
B 316 656	Aug 2 1928	H F Taylor Ass to Atlantic Coast Fisheries	Fish livers are preserved by an oil solution of NaF
B 334 950	Nov 5 1930	E Langefeldt and E Hellerud	Extraction of soap solutions from liver oils by means of benzene introduced in vapor form
B 361 343	Dec " 1931		
B 381 342	Oct 1 st 1932	K. Kawai	Oil from fish livers by treatment with dilute alkali and centrifuging the separated oil
B 392 060	Oct 1 1930	I G	Vitamin-containing hydrogenated fish oils are made aromatic by adding peppermint oil anise oil or the like
B 433 930	Aug 2 nd 1935	Ferrosan A C	Isolation of the vitamin-containing unsaponifiable fraction of oils
B 433 938	Sept 4 1935	I G	Use of hydroxy alkyl-ethers and alkoxy ethyl ethers of polyhydroxy compounds as water miscible solvents for fat soluble vitamins.
B 441 545	Jan 16 1936	B A Rewald	Extraction of vitamins from fish tissue
B 461 70	Feb 1 st 1937	Aarus Olsefabriek and C E Christensen	Isolation of vitamins from oils and fats by simultaneous extraction and saponification
B 494 26	Oct 24 1938	E Aubagen	Manufacture of stable vitamin A and vitamin D preparations by mixing only vitamin preparations with fat-containing plant material e.g. germinated wheat
B 500 770	Feb 15 1939	G H Lubarsky Ass to Vitamol Inc	Manufacture of vitamin compositions for feeding poultry and live stock by emulsifying an oil concentrate of vitamins A and D in molasses
B 506 730	June 5 1939	A E Briod & B R East	Vitamin concentrates and use thereof in making food products particularly milk products
B 533 323	Feb 11 1941	G H Lubarsky Ass to Vitamol Inc	Vitamin composition by mixing a fish liver oil vitamin concentrate emulsified in molasses with an animal feed meal followed by drying
B 535 383	April 8 1941	L O Buxton Ass to National Oil Products	Vitamin concentrate from the unsaponifiable fraction of marine oils by adsorption followed by elution
B 537 403	June 20 1941	L O Buxton and E J Simons Ass to National Oil Products	Refining of fat soluble vitamins containing oils by activated carbon

PATENT No	DATE	PATENTEE	ABSTRACT
Vitamins A and D (Continued)			
B 541 003	Nov 10 1941	H P Kaufmann	Refinement of vitamin-containing oils by chromatographic adsorption
G 452 646	Nov 15 1927	I G	Liver oils are refined by treatment with alcohols in the absence of oxygen
G 468 301	Nov 4 1924	A W Owe	Vitamin extracts <i>e g</i> carotene by saponification neutralization and removal of solvent
G 492 281	June 3 1927	J Wolf	Extraction of livers or fish liver oil with an acidic agent having a pH between 3 and 6
G 448 870	Sept 1 1927	H Sander & Co	Vitamin containing margarine from cod liver oil
G 496 397	April 26 1930		
G 560 146	Feb 26 1926	A G f Medizinische Pro-	Extraction of vitamins from oils by means of hot alcohol and/or acetone
G 567 648	Mar 31 1927	dukte	
G 568 901	Oct 12 1930	J D Riedel E de Haen	Petroleum ether extraction of the saponifiable part of cod liver oil
G 593 395	Feb 26 1934	Pentosin Werke	Emulsion from liver oil lime and calcium saccharate
G 692 711	Sept 27 1940	F Unger Ass to Heyl & Co and to F Unger	Extraction of vitamins A and D by means of esters of low M W acids and low M W alcohols
Belg 430 869	Nov 30 1938	M Vermeulen	Natural products containing vitamins are saponified directly and extracted
Can 259 310	Mar 30 1926	P M Heyerdahl	Incorporation of vitamins in margarine by mixing cod liver oil with olive oil prior to the addition to margarine
Can 374 395	June 14 1938	H N Brocklesby Ass to the Fisheries Research Board of Canada	Process of preparing natural fish liver oils of high vitamin A and D potency by digestion of the liver proteins with pepsin peptization of the digested material with a mild alkali and separation of the liberated oil
Norw 34 895	May 8 1922	T B Lexow	Margarine is enriched in vitamins by the addition of animal fats high in vitamin content such as cod liver oil
Norw 41 688	July 27 1925	Smoerfabrikken Flora	Fish liver oil to be added to margarine is treated to cover the unpleasant taste <i>e g</i> by adding aromatic substances and emulsification
Distillation Procedure			
U S 1 260 072	Mar 19 1918	W P Schuck	Purification of oils by distilling off impurities
B 120 870	Dec 31 1917	Ass to Superior Oil & Process Co	
U S 1 925 559	Sept 5 1933	K C D Hickman	Vacuum distillation of fish oils
U S reissue 20 70	April 26 1938	Ass to Eastman Kodak	

PATENT NO	DATE	PATENTER	ABSTRACT
Distillation Procedure (Continued)			
U S 2 113 30	April 5 1938	K C D Hickman	Process for the separation of vitamins A and D from natural oils by molecular distillation in the presence of dyes as indicator for the distillation points of the vitamins
U S 2 148 7 J	July 26 1938	Ass to Eastman Kodak	
B 479 802	Feb 11 1938		
F 8 5 979	Mar 18 1938		
F 834 540	Nov 23 1938		
U S 2 117 80	May 17 1938	K C D Hickman Ass to Eastman Kodak	Apparatus for molecular distillation of organic substances in degassed condition
U S 2 117 803	May 17 1938	F C D Hickman Ass to Eastman Kodak	Apparatus for molecular distillation by spraying the compound to be distilled under vacuum into the hot vapor of a distilling substance
U S 2 196 466	Aug 9 1938	K C D Hickman Ass to Eastman Kodak	High vacuum short path distillation of oils containing vitamins
U S 1 646 7	Aug 9 1938	K C D Hickman and J C	Short path high vacuum distillation and process for removing the distillate by a flow of an inert liquid over the condensing surface
B 487 697	June 24 1938	Hecker	
F 817 036	Aug 24 1937	Ass to Eastman Kodak	
U S 2 18 73	Aug 30 1938	R C J Fraser Ass to ICI	Fractional short path distillation
U S 2 136 774	Nov 15 1938	K C D Hickman Ass to Distillation Products	Replacing the air by an inert gas or vapor prior to molecular distillation
U S 2 143 587	Jan 10 1939	H I Waterman C van Vlodrop and A VanDijk	Preparation of vitamin A and D concentrates which are free from taste and odor by molecular distillation of fish oils followed by mild hydrogenation
B 452 442	Aug 29 1936		
G 659 917	April 28 1938	Ass to ICI	
F 802 177	Aug 29 1936		
U S 2 144 906	Jan 24 1939	H I Waterman and C van Vlodrop Ass to ICI	Flavoring matters and vitamins from butter fat by distillation
U S 2 146 894	Feb 14 1939	K C D Hickman Ass to Distillation Products	Separation of sterols and vitamin D from oils by high vacuum distillation
U S 2 106 683	Mar 14 1939	K C D Hickman	Distillation of vitamin-containing oils in the presence of antioxidants
B 490 885	Mar 2 1938	Ass to Distillation Products	
F 812 734	May 15 1937		
U S 2 150 684	Mar 14 1939	K C D Hickman	Solid materials containing vitamins are subjected to molecular distillation
F 825 978	Mar 18 1938	Ass to Distillation Products	
U S 2 150 68	Mar 14 1939	K C D Hickman	Apparatus for molecular distillation
B 487 883	Apr 6 1938	Ass to Distillation Products	
U S 2 165 378	July 11 1939	K C D Hickman	Distillation in the presence of a substance of low volatility such as tripelargonine
B 476 134	Dec 2 1937	Ass to Eastman Kodak and to Distillation Products	
B 482 88	Apr 6 1938		
B 490 433	Aug 15 1938		
F 811 766	Apr 22 1937		
F 825 974	Mar 18 1938		
U S 2 169 195	Aug 8 1939	K C D Hickman and A O	Esters of vitamin A and unsaturated fatty acids having at least 8 carbon atoms
B 481 189	Mar 7 1938	Tischer	
G 664 745	Sept 5 1938	Ass to Distillation Products	
U S 2 180 01	Nov 14 1940	K C D Hickman	Method of distilling fish oils to yield products of high vitamin content by subjecting the oil to a degassing operation prior to distillation
U S 2 210 96	Aug 13 1940	Ass to Distillation Products	

PATENT NO	DATE	PATENTEE	ABSTRACT
<i>Distillation Procedure (Continued)</i>			
U S 2 180 356	Nov 21 1939	K C D Hickman Ass to Distillation Products	Distillation of vitamins A and D from fish liver oils
U S 2 186 669	Jan 9 1940	E W Fawcett and G Burrows Ass to ICI	Apparatus for short path high vacuum distillation of vitamin containing fish oils
U S 2 199 994	May 7 1940	K C D Hickman	Distillation of fish oils
U S 2 199 995	May 7 1940	Ass to Distillation Products	
U S 2 205 920	June 25 1940	K C D Hickman Ass to Distillation Products	Concentrates of vitamin A and of vitamins A and D are obtained from naturally occurring animal oils and fats by molecular distillation
U S 2 210 927	Aug 13 1940	K C D Hickman	Vacuum distillation process
G 700 764	Nov 28 1940	Ass to Distillation Products	
F 834 936	Dec 6 1938		
U S 2 229 173	Jan 21 1941	K C D Hickman Ass to Distillation Products	A concentrate of vitamin A and D in ester form is made by molecular distillation of the crude ester asaponification concentration of the vitamin and re esterification
U S 2 249 524	July 15 1941	K C D Hickman and J C Hecker Ass to Distillation Products	Process for removing substances of undesirable odor and taste from vitamin containing oils by short path distillation of the undesirable materials leaving the purified vitamin oil as distillation residue
U S 2 249 525	July 15 1941	K C D Hickman	Purification of vitamin A esters by alcohol extraction of impurities
B 528 994	Nov 21 1940	Ass to Distillation Products	
B 415 088	Aug 17 1934	F H Carr and W Jewell	High vacuum short path distillation of vitamins from unsaponified liver oils
G 670 016	Jan 10 1939	Ass to British Drug Houses	
F 767 191	July 19 1934		Concentration of vitamins by partial saponification followed by high vacuum distillation
B 464 395	April 19 1937	E W Fawcett and D Whitaker	
F 811 920	April 26 1937	Ass to ICI	The undistilled residue of a molecular distillation is recirculated over the vaporizing element of the distillation apparatus
B 479 816	Feb 11 1938	K C D Hickman Ass to Eastman Kodak	
B 487 881	April 6 1938	Eastman Kodak	High vacuum short path distillation in the presence of constant yield oil
B 493 948	Oct 18 1938		
B 485 549	May 18 1938	K C D Hickman Ass to Eastman Kodak	Molecular distillation of unsaturated oils to be used for blending vitamin concentrates
B 487 367	June 20 1938	G G R Smith	Saponification of the vitamin fraction obtained by high vacuum distillation and separation of the unsaponifiable part followed by high vacuum short path distillation
F 834 375	Nov 18 1938	Ass to Eastman Kodak	
B 488 878	July 15 1938	Eastman Kodak	High vacuum distillation of sterols and vitamins
B 489 673	July 29 1938	K C D Hickman and A O Tischer Ass. to Eastman Kodak	High vacuum distillation of materials containing sterols

PATENT NO	DATE	PATENTEE	ABSTRACT
Distillation Procedure (Continued)			
B 500 195 Add to 482 883	July 29 1937	Eastman Kodak	Apparatus for high vacuum distillation
B 501 841	Mar 2 1939	J G Baxter Ass to Eastman Kodak	Distillation of hydrocarbons and vitamins from marine animal oils
B 508 469	June 27 1939	K C D Hickman Ass to Kodak Ltd	Isolation of vitamin A vitamin A esters and vitamin D from fish oils by molecular distillation
B 532 770	Jan 30 1941	K C D Hickman Ass to Distillation Products	Short path high vacuum distillation of vitamins
B 539 089	Aug 27 1941	K C D Hickman Ass to Distillation Products	Extraction of fish tissues with a solvent of a vapor pressure lower than vitamins followed by high vacuum distillation
F 825 406	Mar 3 1938	Eastman Kodak	Process for treating vitamin A consisting in the esterification of the distillate obtained by the molecular distillation of an animal oil containing vitamin A
F 835 973	Mar 18 1938	Eastman Kodak	Cholane derivatives particularly sterols are concentrated by high vacuum short path distillation
F 834 93, F 834 937	Dec 6 1938 Dec 6 1938	Eastman Kodak	Method and apparatus for short path high vacuum distillation of vitamins A and D

VITAMINS A

Provitamins A			
U S 1,328 278	Jan 20 1920	N A Gavin	Extraction of palm oil by cooking centrifuging screening and pressing
U S 1 933 607	April 3 1934	H N Holmes and H M Leicester Ass to S M A Corp	Carotene from green plant material by alkali hydrolysis followed by chloroform extraction
U S 1 967 121	July 17 1934	H N Holmes and H M Leicester Ass to S M A Corp	Carotene from carrots by cooking and acetone extraction
U S 1 978 981	Oct 30 1934	H M Barnett Ass to S M A Corp	Carotene from palm oil by precipitation with iodine
U S 1 988 031	Jan 15 1935	H M Barnett Ass to S M A Corp	Carotene from carrot oil or carrot powder by extraction
U S 2 029 722	Feb 4 1936	V Jersey Ass to S M A Corp	Refining carotene containing oils by separating the free fatty acids present as alkali salts
U S 2 031 991	Feb 25 1936	O Ungnade and W F Richards Ass to S M A Corp	Acetone extraction of carotene from dry soaps
U S 2 032 006	Feb 25 1936	R J Cross Ass to S M A Corp	Addition of water binding materials to carotene-containing soaps and extraction of carotene from the dry material
U S 2 031 165	Feb 5 1936	H M Barnett W O Frohling and A F Germania Ass to S M A Corp	Carotene from carrots or spinach by benzene extraction crystallization of the main amounts of carotene and incorporation of the mother liquor into oil

PATENT NO	DATE	PATENTER	ABSTRACT
Provitamins A (Continued)			
U S 2 131 394	Sept 27 1938	W H Test	Concentrate of pigments from vegetable origin by extraction with an organic solvent from an acidified aqueous solution or suspension
U S 2 170 872	Aug 29 1939	D D Peebles	Macerated fresh plant materials are digested with alkali under pressure followed by solvent extraction
B 304 113	Sept 24 1930	P S Voigt	Provitamin A and Vitamin C are extracted from lemon peel with alcohol
G 07 683	Jan 7 1933	R Kuhn	Separation of alpha and beta carotene by two methods 1 chromatographic adsorption and 2 precipitation with iodine
G 685 390	Dec 16 1939	T Buhr and W Schoenenberger Ass to W Schoenenberger	Provitamin A containing plant juices by centrifuging the plant juice heating the liquid to coagulate and filtering and adding the sediment from the centrifugation to the liquid
Hung 116 057	Mar 16 1937	Ocean Magyar Konzervgyar	Carotene is mixed with pulp of paprika and added to food products
Isolation			
U S 2 076 901	April 13 1937	F Laquer	Preparation of vitamin A by direct saponification of fish livers with aqueous alcoholic caustic alkali
G 634 760	Sept 2 1936	Ass to Winthrop	metal hydroxide followed by extraction
U S 2 111 049	Mar 15 1938	H N Holmes Ass to Parke Davis	Purification of vitamin A by chromatographic adsorption
U S 2 125 215	July 26 1938	A D Barbour	Process for the production of a vitamin containing fish oil partially hydrogenated in the presence of a highly active nickel catalyst
B 500 087	April 25 1938	Ass to Ontario Research Foundation	
B 43 907	Dec 10 1925	Aarhus Oliefabrik and K H Hansen	Incorporation of vitamin A into oil by mixing a vitamin A containing soap from saponified oil with oil and separating the aqueous soap layer
B 83 265	Feb 2 1928	K Kawai	50-75% Saponification of cod liver oils
G 627 912	June 9 1927		
B 306 881	May 19 1930	T Shimizu	Precipitation of vitamin A from aqueous solutions by means of bile acids
F 670 114	Feb 21 1929		
B 393 883	June 10 1933	Hoffmann La Roche	Purification of crude vitamin A preparation by freezing out impurities and precipitating the vitamin A with water The precipitates are filtered through a layer of solid carbon dioxide
C 612 369	April 23 1933		
B 401 095	Nov 9 1933	Abbott	Vitamin A and D concentrates are obtained from fish liver oils by steamizing the livers and extracting the oil with an organic solvent
B 434 432	Sept 2 1933	K Ritvert	Purification of vitamin A in tuna fish liver oil by saponification at room temperature followed by ether extraction
C 636 727	Sept 17 1933	Ass to Merck	
I 768 217	Aug 2 1934		

PATENT NO	DATE	PATENTES	ABSTRACT
<i>Isolation (Continued)</i>			
B 465 547	May 10 1937	K Kawas	Vitamin concentrates from fish oils by saponification followed by extraction with a fatty oil
B 500 645	Not accepted	Aktiebolaget Separator Ass to Bergedorfer Eisenwerk A G Astra Werke	Process and apparatus for the extraction of oil from fish liver by breaking up the cell structure with acids or alkali without dissolving the oil and separating the oil by centrifugal treatment
G 463 301	Nov 10 1928	A W Owe	Partial saponification of plant material while extracting provitamin A
G 540 701	Dec 24 1931	J A de Lourauro	Obtaining vitamin A and vitamin D separately from cod liver oil by extraction with acetic acid followed by petroleum ether extraction of the acetic acid extract Vitamin A is claimed to be in the petroleum ether while vitamin D is in the remaining acetic acid
G 660 621	June 3 1933	Hoffman La Roche	Separation of components of vitamin A by chromatographic adsorption
G 681 730	Sept 29 1939	R Rosenbusch and G Rev erey Ass to Riedel de Haën	Isolation of vitamin A from fish oils by saponification in the presence of minute amounts of water extraction of the solid saponification mass with acetone removal of the acetone by distillation and ether extraction of the residue
G 697 762	Oct 22 1940	R J Wolf Ass to Nordmark Werke	Separation of vitamin A from animal tissues by enzymes followed by extraction with organic solvents
F 847 816	Oct 17 1939	M Vermeulen	Concentration of vitamin A (or provitamins A) by direct saponification in the absence of oxygen extraction of the saponification mass with ether freezing out sterols and distilling off the ether The residual product is claimed to contain from 3,200,000 to 3,600,000 I U vitamin A/gr
Jap 128 808	Feb 15 1939	K Kituta	Concentration of vitamin A of fish liver oil by incomplete saponification (50-75%) and simultaneous extraction
<i>Synthesis</i>			
U S 1 909 110	April 23 1935	L Rusicka	Synthesis of tetrahydro-vitamin A
B 418 723	Oct 30 1934	Ass to Ciba	
G 601 070	Aug 16 1934		
U S 2 175 843	Oct. 10 1939	R Kuhn and C Grundmann Ass to Winthrop	Polyene carboxylic acids and esters and manufacture thereof
U S 2 233 375	Feb 25 1941	R Kuhn and C J O R Morris	Condensing beta ionylidene acetaldehyde in the presence of a secondary amine with beta methyl-crotonaldehyde to form alpha-(beta ionylidene) beta methyl sorbin aldehyde and reducing the aldehyde group of the latter compound by an aluminum salt of a secondary alcohol
G 696 084	Aug 15 1940	Ass to Winthrop	

PATENT NO	DATE	PATENTER	ABSTRACT
Synthesis (Continued)			
U S 2 239 491	April 22 1941	R Kuhn and C J O R Morris Ass to Winthrop	5 (2 Methyl 6 6 dimethyl cyclohexenyl 1') 3 methyl 24-pentadienal is claimed as an intermediate in the synthesis of vitamin A and its preparation from 4 (2 methyl 6 6 dimethyl cyclohexenyl 1) 2 methyl 1,3 butadiene 1-carboxylic acid esters also claimed
B 510 540	Aug 2 1939	I M Heilbron and J W Batty	Condensation of citral with beta methyl croton aldehyde
G 683 030	Oct 27 1939	R Kuhn and K Wallenfels Ass to I G	Production of polyene compounds e g 1 30 diphenylpentadecaene by converting polyene alcohols into thio or seleno-aldehydes and removing from 2 molecules of the latter sulfur or selenium
Analysis			
U S 2 065 953	Dec 29 1937	F Twyman and D H Follet Ass to Hilger	Photometric apparatus for estimating substances such as cod liver oil vitamin A concentrates and fruit juices which have characteristic selective radiation absorption
U S 2 123 573	July 12 1938	R L McFarlan and J W Reddie Ass to United Drug Co	Apparatus for measuring the concentration of vitamin A by light of 330 mμ
F 760 676	Feb 28 1934	Hilger	Determination of vitamin A by light of 328 mμ
Derivatives and Utilization			
U S 2 183 084	Dec 12 1939	S Reynolds Ass to Atlantic Coast Fisheries	Composition of gelatine with vitamin A etc
U S 2 198 214	April 23 1940	S Musher Ass to Musher Foundation	Stabilizing alfalfa against oxidation for the retention of its carotene content during drying and storage by the use of a sugar and a phosphatide or H ₂ PO ₄ in small proportions
U S 2 218 591	Oct 22 1940	H F Taylor Ass to The Atlantic Coast Fisheries	Dispersion of vitamin A in gelatine solution addition of glycerine and subsequent drying
U S 2 218 592	Oct 22, 1940	H F Taylor Ass to Atlantic Coast Fisheries	Substantially dry vitamin A preparations are made by dispersing the oil containing the vitamin in a matrix composed of a gelable colloid e g gelatine and an invert sugar e g molasses honey etc
B 491 212	Aug 24 1938	S Reynolds Ass to Glaxo	Process for the production of drop lets of gelatine containing vitamin A
B 503 517	Not accepted	J Verne and C Mille	Process of protecting fatty substances from becoming rancid by the addition of carotenoids
B 507 471	June 12 1939	Eastman Kodak	Retarding oxidation of animal and vegetable oils by the addition of a small quantity of a fraction possessing antioxidant properties taken from the first 20% distillate obtained by a high vacuum short path distillation of vegetable oils.

PATENT NO	DATE	PATENTEE	ABSTRACT
Derivatives and Utilization (Continued)			
G 658 957	April 20 1938	I G	Preparation of an emulsion of fat soluble vitamin concentrates from fish livers

VITAMIN B COMPLEX

U S 1 058 927	April 15 1913	J Tsuzuki Japan	Isolation of vitamin B concentrates from rice bran by alcohol extraction
G 266 211	Feb 6 1911		
B 5322	Nov 12 1911		
U S 1 162 908	Dec 7 1915	C Funk	Extraction of vitamin B from yeast rice bran etc by addition of phosphotungstic acid and extraction with acetone
U S 1 173 317	Feb 29 1918	A Seidell	Vitamins from yeast by autolysis and adsorption on fuller's earth followed by elution with dilute acid and alcohol
U S 1 235 198	July 31 1917	A Gams and B Schreiber	Vitamin concentrate from yeast rice bran beans etc by precipitation of inactive materials with lead salts and precipitation of the active material with an alkaloid precipitant such as phosphotungstic acid
U S 1 431 575	Oct 10 1922	C Hoffman II D Grigsby	Addition of vitamin B containing substances such as defatted rice polishings to dough
B 186 633	Oct 9 1922	and N M Gregor Ass to Ward Baking Co	
U S 1 474 029	Nov 13 1923	I F Harris	Alcohol extraction of vitamins from yeast boiled in dilute acetic acid
U S 1 617 70	Feb 15 1927	P Caccia	Extraction of vitamin B with alcohol precipitation with ethylene dichloride and crystallization in the presence of sulfuric acid
U S 1 737 70	Nov 26 1930	L Wallerstein Ass to Wallerstein & Co	Manufacture of extracts rich in vitamin B from malt and wheat germs
U S 1 842 033	Jan 26 1932	B W Dedrick	Water extraction of the residues obtained by making flour from wheat followed by filtration and recovery of the water soluble materials
U S 1 893 977	Jan 31 1933	J W Dressel	Liver concentrate by extraction with water
B 373 038	Sept 1 1931		
U S 2 006 033	June 25 1935	F Lange and L Taube Ass to Winthrop	Preparations containing vitamin B from yeast extract
U S 2 006 699	July 2 1935	G C Supplee and G E Flanigan Ass to The Borden Co	Recovery of vitamins B ₁ and B ₂ from milk
U S 2 095 638	Oct 12 1937	G A Jeffreys	Liquid binder for food products rich in vitamin B from fermented material
U S 2 184 748	Dec 26 1939	R F Light and C N Frey	Preparation of vitamins B ₁ and B ₂ from yeast by plasmolysis coagulation of the protein material and concentration of the water solution
B 428 044	Apr 130 193	Ass to Standard Brands	

PATENT NO	DATE	PATENTEE	ABSTRACT
U S 2 193 876	Mar 19 1940	B Maize) Ass to Vico Products Co	Isolation of the vitamin B complex from yeast by means of 90% m or ethyl alcohol distilling off of the solvent to separate its constituents or precipitating by alkali earth materials and using the vitamin-containing material with extracted yeast or with isolated egg white
U S 2,202 161	May 28 1940	C S Miner Ass to Commercial Solvents	Recovery of vitamin B complex from fermentation of molasses by butyl alcohol producing bacteria
U S 2 202 307	May 28 1940	L E Booher	Extraction of the vitamin B complex
B 103 294 F 485 649	Jan 4 1917 Jan 24 1918	Ciba	Extraction of vitamins from husks yeast etc by alcohol and precipitation of inactive products by lead salts
B 345 689 G 566 174	Nov 22 1929 Oct 10 1929	I G	Aqueous solution of yeast rich in vitamin B for beverages
B 376 149 Jap 91 200	Nov 10 1931 April 28 1931	S Ota and K Umeda Ass to K Beer	Pressed yeast is fermented with cane sugar etc The product is extracted with alcohol to yield a nutrient material containing vitamin B
B 434 058	Aug 26 1935	W W Triggs	Concentrate of vitamins B from whey
B 477 528	Dec 30 1937	Standard Brands	Manufacture of a product containing vitamins of the B-complex by extraction of seeds or grain germs
B 485 079 F 824 837	May 10 1938 Feb 17 1938	I G	Plant growth stimulant containing vitamins of the B-complex
B 486 054	May 30 1938	El Lilly	Solution of a vitamin of the B-complex in a polyhydric alcohol
B 536 510	May 16 1941	Standard Brands	Yeast is grown on a culture medium containing the vitamins B ₁ B ₂ , inositol pantothenic acid and biotic acid (extracted from sheep liver)
B 538 191	July 24 1941	Emulsions Process Corp	Extraction of the vitamins of the B-complex from yeast after breaking down the yeast cells by application of pressure followed by a sudden release of the pressure
B 539 825	Sept 25 1941	Standard Brands	Improvement in the propagation of yeast by the addition to the culture medium of vitamins B ₁ and B ₂ (or a pyrimidine and thiazol compound) and additional growth promoting factors
G 295 381	Mar 25 1914	Boehringer	Purification of phosphotungstate precipitates of vitamins B by acetone extraction
G 607 512 G 634 969	Dec 29 1934 Sept 10 1936	P György R Kuhn and T Wagner Jahrgg Ass to I G	Manufacture of vitamins of the B complex by adsorption followed by elution with amines or ammonia

PATENT NO	DAYS	PATENTEE	ABSTRACT
G 630 772	June 5 1936	I G	Isolation of vitamin B ₁ by adsorption elution and precipitation methods making use of the strong bluish fluorescence characteristics of vitamin B
G 646 548 Continuation of G 630 772	June 17 1937	I G	Elution of vitamin B by alkali and by amines. Vitamin B ₁ according to this patent has the formula C ₁₂ H ₁₇ N ₄ O ₄ S and seems to resemble vitamin B ₁ and thiochrome rather than the description of vitamin B ₁
G 672 078 Continuation of 661 929 and 670 749	Feb 18 1939	I G	Method of concentrating growth promoting compounds of the B ₁ type
G 703 400	Mar 7 1941	Ass to G Henning	Adenosinphosphoric acid from adenosin and phosphoric acid by yeast
Jap 91 202	April 25 1931	S Izume M Sato I Seto Ass to the South Manchurian Railway Co	The alcoholic extract from soy bean is freed from impurities and contains large amounts of vitamin B
Jap 101 137	May 16 1933	K Taguchi Ass. to K Katakura	Rice bran or the like is extracted with methanol to yield a substance rich in vitamin B

VITAMIN B₁—THIAMIN

Isolation			
U S 1889 721	Aug 2 1932	B Sure	Purification of crude vitamin B ₁ concentrates by dissolving in acetic acid precipitating impurities with acetone and adsorbing the vitamin on charcoal
U S 1889 427	Nov 29 1932		
B 354 421	Aug 13 1931		
F 714 416			
U S 1937 671	Dec 5 1933	A Seidell	Elimination of non vitamin B ₁ substances from vitamin B ₁ concentrates by benzoylation
U S 1990 961	Feb 12 1935	E H Stuart Ass to Eli Lilly	Removal of vitamin B ₁ from an adsorbate by solutions of mineral acids
U S 2007 519	May 28 1935	R J Block and G R Cowgill Ass to S J Dannenberg	Purification of vitamin B ₁ concentrates by oxidation of impurities
U S 2015 876	Oct 1 1935	B Sure and E H Stuart Ass to B Sure	Concentration of vitamin B from acid alcoholic extracts from rice brans by adsorption on active carbon
U S 2049 988	Aug 4 1936	R R Williams and R E Waterman Ass to Research Corp	Elution of the fuller's earth adsorbate of vitamin B ₁ with an acid polynitrogenous alkaloid salt and with quinine sulfate solution
U S 2114 775	April 19 1938	L R Cerecedo	Isolation of vitamin B ₁ from natural sources by adsorption on zeolite followed by elution. Further purification by precipitation of the vitamin as silver compound or silicotungstate
B 497 081	Not accepted		
F 818 702	Oct 2 1937		
B 390 378	April 6 1933	S J Dannenberg	Vitamin B concentrate by extracting the vitamin at pH 10-13 followed by an acid extraction

PATENT NO	DATE	PATENTEE	ABSTRACT
<i>Isolation (Continued)</i>			
G 311 074	Jan 12 1918	Ciba	Extraction of animal or plant material with dilute alcohol and precipitation of impurities with less salts
G 370 785	April 28 1920	R. Bosshard and P. Hefti	Vitamin B ₁ concentrates from plant material & 2 rice brans yeast etc by total hydrolysis with dil mineral acids at 80° C
G 359 878 Continuation of G 311 074	Sept 28 1922	Ciba	Hydrolysis of the starting material with enzymes
Jap 109 268	Jan 22 1935	R. Otake	Isolation of crystallized vitamin B ₁ from yeast by adsorption on acid clay extraction with Ba(OH) ₂ and precipitation with a silver salt.
<i>Synthesis</i>			
U S 2 127 446	Aug 16 1938	M. Klingensuss	Synthesis of vitamin B ₁ by condensation of 2 methyl-4 amino-5-thioformylamino methyl pyrimidine with 2 methyl 2 alkoxy-3-chlorotetra hydro-furane
B 500 519	Feb 10 1939	Ass to Hoffmann LaRoche	
G 676 980	June 16 1939		
F 831 110	Aug 23 1938		
U S 2 166 233	July 18 1939	E. R. Buchman Ass to Research Corp	Process of preparing 2 methyl-6-amino-5-pyrimidyl bromoacetic acid by bromination of the corresponding pyrimidine acetic acid and method of synthesizing vitamin B ₁ therefrom by condensation with 4 methyl 5 beta hydroxy ethyl thiazole
U S 2 184 720	Dec 26 1939	T. Matukawa and M. Ohta Ass to Kabusiki Kaisha Ta keda Chobei Shoten	Synthesis of vitamin B ₁ from 2 methyl 4 amino 5 formamino-methyl pyrimidine and gamma gamma-diaceto gamma mercapto-propyl alcohol
U S 2 209 244	July 23 1940	H. Andersag and K. Westphal	(1) Synthesis of vitamin B ₁ by condensation of 2 methyl-4 amino-5-thio formylamidomethyl pyrimidine with 2 keto-3 halogeno-pentanol 5 or its acetate (2) Synthesis of vitamin B ₁ by condensation of 4 methyl 5 beta hydroxy ethyl thiazole with 2 methyl 4 amino 5 halogeno methyl pyrimidine (3) Synthesis of vitamin B ₁ by condensation of an ester of gamma (4 methyl 5 acyloxy ethyl N thiazolium bromide) alpha cyano-butyric acid with acetamidine
B 456 735	Nov 11 1936	Ass to I G	
B 471 416	Aug 30 1937		
G 685 032	Dec 11 1939		
F 816 432	Aug 7 1937		
U S 2 235 862	Mar 20 1941	O. Zima	Synthesis of vitamin B ₁ by condensation of 2 methyl-4 amino-5-oxymethyl pyrimidine HCl with 4 methyl 5 beta hydroxy ethyl thiazole HCl
B 507 918	June 22 1939	Ass to Merck	
G 669 187	Dec 19 1938		
Continuation of G 681 638			
U S 2 252 921	Aug 19 1941	Z. Földi and A. Csercs	Synthesis of vitamin B ₁ from 2 methyl 4 amino 5 thioformamidomethyl pyrimidine and 2 methyl 2 hydroxy 3 halogenotetrahydrofuran in the presence of salts of weak organic bases and strong acids

PATENT NO	DATE	PATENTEE	ABSTRACT
Synthesis (Continued)			
496 796	Dec 2 1938	Research Corp	Synthesis of vitamin B ₁ by condensation of 2 methyl 4 amino 5-bromo-methyl pyrimidine HBr with 4 methyl 5 (beta hydroxy ethyl) thiazole
834 68	Nov '9 1939		
53 013	Jan 15 1941	Standard Brands and International Yeast	The vitamin B ₁ content of yeast is increased by growing the yeast in a wort to which has been added a pyrimidine compound with or without a thiazole compound 2 Methyl 5 ethoxymethyl 6-amino-pyrimidine and 4 methyl 5-beta hydroxy-ethyl thiazole are specifically mentioned as additive agents
681 638	Sept '7 1939	O Zima Ass to Merck	Synthesis of vitamin B ₁ by condensation of 2 methyl-4-amino-5-alkoxy methyl pyrimidine hydrochloride with the hydrochloride of 4 methyl 5 beta hydroxy ethyl thiazole.
703 775	Mar 15 1941	H Andersag and K. Westphal Ass to I G	Synthesis of vitamin B ₁ according to G P 685 032 but using 5-amino-alkyl thiazole compounds for the condensation followed by conversion into vitamin B ₁
705 432	April 28 1941	K Westphal and H Andersag Ass to I G	Synthesis of vitamin B ₁ by condensation of 4 methyl 5 beta hydroxy ethyl thiazole with 2 methyl-4-amino-5-methyl-pyrimidine the 5-methyl group of which is substituted by a reactive group (Example benzol sulfonic acid ester of the 5-hydroxy methyl derivative)
Synthesis of the Thiazole Part			
123 653	July 12 1938	Hoffmann LaRoche	2 Methyl 2 alkoxy 3 chlorotetra hydro-furane from alpha-acetyl alpha chloro butyrolactone and an aliphatic primary alcohol
496,801	Dec 6 1938		
684,587	Dec 1 1939		
831 111	Aug 23 1938		
2 133 979	Oct ' 1938	E R B chman Ass to Research Corp	4 Methyl 5 beta hydroxy ethyl thiazole from gamma aceto gamma halogeno-propanol or thio-formamide
472 459	Sept 17 1937		
673 174	Mar 17 1939		
672 617	July 21 1939		
803 495	Oct. 1 1936		
2 134 015	Oct. 20 1938	R R Williams Ass to Research Corp	Composition of matter claims for methyl 5 beta hydroxy ethyl thiazole and its salts
2 139,570	Dec 6 1938	H Andersag and K. Westphal Ass to Winthrop	Condensation of gamma-bromo gamma aceto propanol with a rhodamide followed by treatment to form a methyl thiazolyl 5 esters
468 751	Nov 13 1937		
704,206	Mar 26 1941		
811,224	April 9 1937		
2 160,867	June 6 1939	O Hromatka Ass to Merck	4 Methyl 5 beta ethyl thiazole from gamma-gamma-aceto-propyl formamide and sulfide
670 131	Jan. 12 1939		

PATENT NO	DATE	PATENTEE	ABSTRACT
Isolation (Continued)			
G 311 074	Jan 12 1916	Ciba	Extraction of animal or plant material with dilute alcohol and precipitation of impurities with lead salts
G 320 785	April 28 1920	R. Bosshard and F. Hefti	Vitamin B ₁ concentrates from plant material e. g. rice brans yeast etc. by total hydrolysis with dil. mineral acids at 80° C
G 359 878 Continuation of G 311 074	Sept 28 1922	Ciba	Hydrolysis of the starting material with enzymes
Jap 109 268	Jan 29 1935	R. Otake	Isolation of crystallized vitamin B ₁ from yeast by adsorption on acid clay extraction with Ba(OH) ₂ and precipitation with a silver salt
Synthesis			
U S 2 127 446	Aug 16 1938	M. Klingenfuss	Synthesis of vitamin B ₁ by condensation of 2 methyl 4 amino 5 thioformylamino methyl pyrimidine with 2 methyl 2 alkoxy 3-chloro tetra hydro-furane
B 500 519	Feb 10 1939	Ass to Hoffmann LaRoche	
G 676 980	June 16 1939		
F 831 110	Aug 23 1938		
U S 2 186 233	July 18 1939	E. R. Buchman Ass to Research Corp	Process of preparing 2 methyl-6 amino-5 pyrimidyl bromoacetic acid by bromination of the corresponding pyrimidine acetic acid and method of synthesizing vitamin B ₁ therefrom by condensation with 4-methyl 5 beta hydroxy ethyl thiazole
U S 2 184 720	Dec 26 1939	T. Matukawa and M. Ohta Ass to Kabushiki Kaisha Ta keda Chobei Shoten	Synthesis of vitamin B ₁ from 2 methyl 4 amino 5 formamino-methyl pyrimidine and gamma gamma-diaceto gamma mercapto-propyl alcohol
U S 2 209 244	July 23 1940	H. Andersag and K. Westphal	(1) Synthesis of vitamin B ₁ by condensation of 2 methyl 4 amino-5 thio formylamidomethyl pyrimidine with 2 keto-3 halogeno pentanol 5 or its acetate (2) Synthesis of vitamin B ₁ by condensation of 4 methyl 5 beta hydroxy ethyl thiazole with 2 methyl 4 amino 5 halogeno methyl pyrimidine (3) Synthesis of vitamin B ₁ by condensation of an ester of gamma (4 methyl 5 acyloxy ethyl N thiazolium bromide) alpha cyano-butyric acid with acetamidide
B 456 735	Nov 11 1936	Ass to J. G.	
B 471 416	Aug 30 1937		
G 685 032	Dec 11 1939		
F 816 439	Aug 7 1937		
U S 2 235 862	Mar 25 1941	O. Zima	Synthesis of vitamin B ₁ by condensation of 2 methyl 4 amino 5 oxy methyl pyrimidine HCl with 4 methyl 5 beta hydroxy ethyl thiazole HCl
B 507 918	June 29 1939	Ass to Merck	
G 669 187	Dec 19 1938		
Continuation of G 681 638			
U S 2 259 921	Aug 19 1941	Z. Földi and A. Gerecs	Synthesis of vitamin B ₁ from 2 methyl 4 amino 5 thioformamidomethyl pyrimidine and 2 methyl 2 hydroxy 3 halogeno tetrahydrofuran in the presence of salts of weak organic bases and strong acids

PATENT NO	DATE	PATENTEE	ABSTRACT
Synthesis (Continued)			
B 496 796 F 834 68	Dec 2 1938 Nov 29 1938	Reston Corp	Synthesis of vitamin B ₁ by condensation of 2 methyl 4 amino 5-bromo methyl pyrimidine HBr with 4 methyl 5 (beta hydroxy ethyl) thiazole
B 53 013	Jan 15 1941	Standard Brands and International Yeast	The vitamin B ₁ content of yeast is increased by growing the yeast in a wort to which has been added a pyrimidine compound with or without a thiazole compound 2 Methyl 5 ethoxymethyl 6 amino-pyrimidine and 4 methyl 5-beta hydroxy ethyl thiazole are specifically mentioned as additive agents
G 681 638 Continuation of G 689 187	Sept 27 1939	O Zima Ass to Merck	Synthesis of vitamin B ₁ by condensation of 2 methyl 4 amino-5-alkoxy methyl pyrimidine hydrochloride with the hydrochloride of 4 methyl 5 beta hydroxy ethyl thiazole
G 703 775 Addition to G 685 032	Mar 10 1941	H Andersag and K Westphal Ass to I G	Synthesis of vitamin B ₁ according to G P 685 032 but using 5-amino alkyl thiazole compounds for the condensation followed by conversion into vitamin B
G 705 432 Addition to G 685 032	April 28 1941	K Westphal and H Andersag Ass to I G	Synthesis of vitamin B ₁ by condensation of 4 methyl 5 beta hydroxy ethyl thiazole with 2 methyl-4 amino-5 methyl pyrimidine the 5 methyl group of which is substituted by a reactive group (Example benzol sulfonic acid ester of the 5-hydroxy methyl derivative)
Synthesis of the Thiazole Part			
U S 2 123 653 B 496 801 G 684 587 F 831 111	July 12 1938 Dec 6 1938 Dec 1 1939 Aug 23 1938	Hoffmann LaRoche "	4 Methyl 5 beta hydroxy ethyl thiazole from gamma aceto-gamma halogeno propanol and thio-formamide
U S 2 133 969 B 472 459 C 673 174 G 670 617 F 803 493	Oct 25 1938 Sept 17 1937 Mar 17 1939 July 21 1939 Oct 1 1938	E R Buchman Ass to Research Corp	Composition of matter claims for 4 methyl 5 beta hydroxy ethyl thiazole and its salts
U S 2 134 015	Oct 20 1938	R R Williams Ass to Research Corp	Condensation of gamma bromo gamma aceto propanol esters with a rhodanide followed by acid treatment to form 2 hydroxy-4 methyl thiazolyl 5 ethanol esters
U S 2 139 570 B 456 751 G 704 236 F 811 224	Dec 6 1939 Nov 13 1936 Mar 28 1941 April 9 1937	H Andersag and K Westphal Ass to W anthrop	4 Methyl 5 beta hydroxy ethyl thiazole from gamma halogeno gamma aceto-propyl alcohol formamide and phosphorus pentasulfide
U S 2 160 867 G 670 131	June 6 1939 Jan 12 1939	O Hromatka Ass to Merck	

PATENT No	DATE	PATENTEE	ABSTRACT
Isolation (Continued)			
G 311 074	Jan 1 st 1916	Ciba	Extraction of animal or plant material with dilute alcohol and precipitation of impurities with lead salts
G 3 rd 785	April 28 19 th 0	R. Bosshard and P. Hefti	Vitamin B ₁ concentrates from plant material & rice bran yeast etc by total hydrolysis with d l mineral acids at 80° C
G 359 878 Continuation of G 311 074	Sept 28 19 th 2	Ciba	Hydrolysis of the starting material with enzymes
Jap 109 268	Jan 22 1935	R. Otake	Isolation of crystallized vitamin B ₁ from yeast by adsorption on acid clay extraction with Ba(OH) ₂ and precipitation with a silver salt
Synthesis			
U S 2 127 446	Aug 16 1938	M. Klingenfuss	Synthesis of vitamin B ₁ by condensation of 2 methyl 4 amino 5 thioformylamino methyl pyrimidine with 2 methyl 2 alkoxy 3 chloro tetra hydro furane
B 500 519	Feb 10 1939	Ass to Hoffmann LaRoche	
G 676 980	June 16 1939		
F 831 110	Aug 23 1938		
U S 2 166 233	July 18 1939	E R. Buchman Ass to Research Corp	Process of preparing 2 methyl-6-amino 5 pyrimidyl bromoacetic acid by bromination of the corresponding pyrimidine acetic acid and method of synthesizing vitamin B ₁ therefrom by condensation with 4 methyl 5 beta hydroxy ethyl thiazole
U S 2 184 720	Dec 26 1939	T. Matukawa and M. Ohta Ass to Kabushiki Kaisha Ta keda Chobei Shoten	Synthesis of vitamin B ₁ from 2 methyl 4 amino 5 formamido methyl pyrimidine and gamma gamma-diaceto gamma mercapto propyl alcohol
U S 2 209 244	July 23 1940	H. Andersag and K. Westphal	(1) Synthesis of vitamin B ₁ by condensation of 2 methyl 4 amino-5 thio formylamidomethyl pyrimidine with 2 keto 3 halogeno pentanol 5 or its acetate (2) Synthesis of vitamin B ₁ by condensation of 4 methyl 5 beta hydroxy ethyl thiazole with methyl 4 amino 5 halogeno methyl pyrimidine (3) Synthesis of vitamin B ₁ by condensation of an ester of gamma (4 methyl 5 acyloxy ethyl N thiazolium bromide) alpha cyano-butyric acid with acetamidine
B 456 735	Nov 11 1936	Ass to I G	
B 471 416	Aug 30 1937		
G 685 032	Dec 11 1939		
F 816 43 rd	Aug 7 1937		
U S 2 235 862	Mar 25 1941	O. Zima	Synthesis of vitamin B ₁ by condensation of 2 methyl 4 amino-5 oxy methyl pyrimidine HCl with 4 methyl 5 beta hydroxy ethyl thiazole HCl
B 507 918	June 2 nd 1939	Ass to Merck	
G 669 187	Dec 19 1938		
Continuation of G 681 638			
U S 2 252 921	Aug 19 1941	Z. Földi and A. Gerecs	Synthesis of vitamin B ₁ from 2 methyl 4 amino 5 thioformamidomethyl pyrimidine and 2 methyl 2 hydroxy 3 halogeno tetrahydrofuran in the presence of salts of weak organic bases and strong acids

PATENT NO	DATE	PATENTEE	ABSTRACT
Synthesis (Continued)			
B 496 796 F 834 68	Dec 1938 Nov 29 1938	Research Corp	Synthesis of vitamin B ₁ by condensation of 2 methyl 4 amino 5-bromo-methyl pyrimidine HBr with 4 methyl 5 (beta hydroxy ethyl) thiazole
B 539 013	Jan 15 1941	Standard Brands and International Yeast	The vitamin B ₁ content of yeast is increased by growing the yeast in a wort to which has been added a pyrimidine compound with or without a thiazole compound 2 Methyl 5 ethoxymethyl 6 amino-pyrimidine and 4 methyl 5 beta hydroxy ethyl thiazole are specifically mentioned as additive agents
G 681 638 Continuation of G 669 187	Sept 27 1939	O Zima Ass to Merck	Synthesis of vitamin B by condensation of 2 methyl-4 amino 5 alkoxy methyl pyrimidine hydrochloride with the hydrochloride of 4 methyl 5 beta hydroxy ethyl thiazole
G 703 775 Addition to G 685 032	Mar 15 1941	H Andersag and K Westphal Ass to I G	Synthesis of vitamin B ₁ according to G P 685 032 but using 5-amino alkyl thiazole compounds for the condensation followed by conversion into vitamin B
G 705 432 Addition to G 685 039	April 28 1941	K Westphal and H Andersag Ass to I G	Synthesis of vitamin B ₁ by condensation of 4 methyl 5 beta hydroxy ethyl thiazole with 2 methyl-4 amino 5 methyl pyrimidine the 5-methyl group of which is substituted by a reactive group (Example benzol sulfonic acid ester of the 5-hydroxy methyl derivative)
Synthesis of the Thiazole Part			
U S 2 123 653 B 496 801 G 684 587 F 831 111	July 12 1938 Dec 6 1938 Dec 1 1939 Aug 23 1938	Hoffmann La Roche	2 Methyl 2 alkoxy 3 chloro tetra hydro furane from alpha acetyl alpha chloro butyric ester and an aliphatic primary alcohol
U S 2 133 969 B 4 459 G 673 174 G 673 617 F 803 495	Oct 25 1938 Sept 17 1937 Mar 17 1939 July 21 1939 Oct 1 1936	E R Buchman Ass to Research Corp	4 Methyl 5 beta hydroxy ethyl thiazole from gamma aceto-gamma halogeno-propanol and thio-formamide
U S 2 134 015	Oct 25 1938	R R Williams Ass to Research Corp	Composition of matter claims for 4 methyl 5 beta hydroxy ethyl thiazole and its salts
U S 2 139 570 B 456 751 G 704 736 F 811 224	Dec 6 1938 Nov 13 1936 Mar 26 1941 April 9 1937	H Andersag and K. Westphal Ass to Winthrop	Condensation of gamma bromo gamma aceto propanol esters with a rhodanide followed by acid treatment to form 2 hydroxy-4 methyl thiazolyl 5 ethanol esters
U S 160 867 G 670 131	June 6 1939 Jan 12 1939	O Hromatka Ass to Merck	4 Methyl 5 beta hydroxy ethyl thiazole from gamma halogeno gamma aceto-propyl alcohol formamide and phosphorus pentasulfide

PATENT NO	DATE	PATENTEE	ABSTRACT
Synthesis of the Thiazole Part (Continued)			
U S 2 179 984 B 492 637 G 678 153	Nov 14 1939 Sept 23 1938 July 13 1939	H Spiegelberg Ass to Hoffmann LaRoche	4 Methyl 5 beta hydroxy ethyl thiazole from the corresponding 2 mercapto derivative by hydrogen peroxide oxidation
U S 2 193 858	Mar 19 1940	E R Buchman Ass to Research Corp	Composition of matter claims for alpha halogeno alpha aceto gamma butyrolactone and method of preparation by halogenation of alpha aceto gamma butyrolactone
U S 2 194 179 Continuation of U S 2 133 969	Mar 19 1940	E R Buchman Ass to Research Corp	4 Methyl 5 beta acetoxy ethyl thiazole from gamma halogeno gamma aceto propyl lactate and thio-formamide
U S 2 209 092	July 23 1940	D Price and I D Pickel Ass to National Oil Products	Preparation of 4 methyl 5 beta amino-ethyl thiazole by brominating levulinic ester reacting the halogenated compound with thio-formamide treating the reaction product with ammonia dehydrating the amide thus obtained to the corresponding cyano-derivative and catalytically hydrogenating the cyano-group to an amino-group
U S 2 216 574 B 472 396	Oct 1 1940 Sept 17 1937	T D Perrine Ass to Research Corp	Halogeno aceto-propyl alcohol from aceto-propyl alcohol by direct halogenation
U S 2 218 349	Oct 15 1940	E R Buchman Ass to Research Corp	Composition of matter claims for gamma halogeno-gamma aceto-propyl alcohol and methods of preparation by halogenation of gamma aceto-propyl alcohol
U S 2 218 350	Oct 15 1940	E R Buchman Ass to Research Corp	Method of producing gamma halogeno-gamma aceto propyl alcohol by simultaneous hydrolysis and decarboxylation of an alpha halogeno-alpha aceto-gamma butyrolactone
U S 2 223 885	Dec 3 1940	I R Buchman Ass to Research Corp	gamma Halogeno gamma aceto propyl esters and their preparation by halogenation of a gamma aceto propyl ester
U S 2 263 014	Nov 18 1941	W Scott Ass to Wingfoot Corp	Preparation of thiazoles from gamma mercapto-thiazoles by pyrolysis
U S 2 267 313 B 490 571 F 876 067	Dec 23 1941 Aug 17 1938 Mar 22 1938	J R Stevens and G A Stein Ass to Research Corp Research Corp	Production of gamma aceto propyl ether Process for the manufacture of gamma halogeno gamma aceto propyl alcohol and of alpha halogeno-alpha aceto-gamma butyrolactone from alpha aceto-gamma butyrolactone
G 664 789	Sept 5 1938	A Wenz Ass to Merck	4 Methyl 5 beta hydroxy ethyl thiazole by halogenation of alpha aceto-butyrolactone followed by condensation with thio-formamide

PATENT NO.	DATE	PATENTEE	ABSTRACT
Synthesis of the Thiazole Part (Continued)			
G 702 831	Feb 17 1941	H Andersag and K. Westphal Ass to I G	4 Methyl 5 beta hydroxy ethyl thiazole from gamma aceto-gamma halogeno propyl compounds and thioformamide followed by saponification
G 705 434	April 28 1941	K. Westphal and H Andersag Ass to I G	2 Methyl 4 alkoxy 3 halogeno-tetra hydro-furane from 1 aceto 1 halogeno 1 alkoxy ethyl acetone and a mineral acid
Synthesis of the Pyrimidine Part			
U S 2 153 016	April 4 1939	R R Williams Ass to Research Corp	2 Methyl 6 amino methyl 6 amino-pyrimidine and derivatives
U S 2 184 964	Dec 26 1939	G A Stein Ass to Research Corp	2 Methyl 5 chloromethyl 6-amino-pyrimidine from the corresponding 5 alkoxy compound
U S 2 194 190	Mar 19 1940	R R Williams Ass to Research Corp	2 Methyl 5 methyl 4 halogeno-pyrimidines
U S 2,220 243	Nov 5 1940	M Hoffer Ass to Hoffmann LaRoche	2 Methyl 4 amino 5 thioformyl amino methyl pyrimidine and its manufacture from 2 methyl 4 amino 5 amino methyl pyrimidine dihydrochloride and potassium di thioformate
B 478 993	Jan 28 1938		
G 675 831	May 20 1939		
F 822 533	Dec 31 1937		
U S * 235 638	Mar 18 1941	O Hromtka	2 Methyl 4 amino 5 cyano-pyrimidine from amino-methylene malonitrile and acetamino-ethyl ether
G 667 990	Nov 24 1938	Ass to Merck	
B 473 193	Oct 4 1937	H Andersag and K. Westphal	2 Methyl 4 amino 5 amino-methyl pyrimidine from acetamidene and formyl malonic ester
G 670 090	Jan 11 1939	Ass to I G	
F 819 596	Oct 21 1937		
B 475 507	Nov 19 1937	I G	4 Amino 5 hydroxy alkyl pyrimidines from the corresponding 5 amino-compound by nitrous acid
B 475 509	Nov 19 1937	H Andersag and K Westphal	Synthesis of 2 methyl-4 amino-methyl pyrimidine by condensation of acetamidene with alpha-cyano-succinic ester to the corresponding 6 hydroxy pyrimidine derivative and elimination of the 6-hydroxyl group via the chloride
G 671 787	Feb 15 1939	Ass to I G	
B 486 414	June 2 1938	Hoffmann LaRoche	Manufacture of 2 methyl-4 amino-5-cyano-pyrimidine from acetamino-ethyl-ether and amino methylene-malonitrile
B 496 738	Dec 2 1938	Research Corp	2 Methyl 4 amino 5 hydroxy methyl pyrimidine by condensation of acetamidine with formyl beta ethoxy propionic ester followed by chlorination and replacement of the chlorine by an amino group
B 52 531	June 20 1940	Merck	2 Methyl 5 chloro methyl 4 amino-pyrimidine from the corresponding 5-alkoxy-compound by hydrogen chloride

PATENT NO	DATE	PATENTER	ABSTRACT
Synthesis of the Pyrimidine Part (Continued)			
B 538 743	Aug 14 1941	Chinoin Gyogyszer es Vegyesszeti Termekek	2 Alkyl 4 amino 5 carbal koxypyrimidines from alpha-cyanobeta amidino acrylic acid esters
G 670 635	Jan 23 1939	O Hromatka Ass to Merck	2 Methyl 4 amino 5 cyano-pyrimidine from alkoxy methylene malonic acid derivatives ammonia and acetimido ethyl ether
Derivatives and Utilization			
U S 2 188 323	Jan 30 1940	H Tauber	Synthesis of cocarboxylase from vitamin B ₁ pyrophosphate and phosphoric acid
G 704 172	Mar 25 1941	Ass to Merck	
U S 2 205 807	June 25 1940	J Bjorksten	Composition of matter claims for vitamin B ₁ and biotin in a solution of a pH below 4.5 for the thiazole and pyrimidine parts of the vitamin B ₁ molecule either separately or together in the presence of biotin at a pH between 2 and 4.5 as plant growth stimulants
U S 2 224 174	Dec 10 1940	J Weijlard Ass to Merck	Purification of synthetic cocarboxylase from vitamin B ₁ by fractional precipitation of the silver salts at various pH
G 663 588	Aug 9 1938	K Lohmann and P Schuster Ass to I G	Isolation of cocarboxylase
Swed 94 746	Feb 22 1939	H v Euler and R Vestin	Vitamin B ₁ phosphate by enzymatic phosphorylation of the vitamin
Swiss 197 717	Aug 1 1938	Hoffmann LaRoche	HCl salts of vitamin B ₁ by precipitating a picrate and converting the latter to the HCl salt

VITAMIN B₁—RIBOFLAVIN

Isolation			
U S 2 139 857	Dec 13 1938	H F Seibert Ass to S M A Corp	Precipitation of riboflavin in mixture with a precipitation of lead sulfide followed by extraction of the filtered sulfide
U S 2 170 014	Oct 3 1939	L E Booher and L T Work	Isolation of riboflavin from adsorbates by elution with water alcohol or acetic acid preferably at an elevated temperature
U S 2 186 314	Jan 9 1940	S Ausbacher G E Flanagan and G C Supplee Ass to The Borden Co	Extraction of riboflavin adsorbates with aqueous acetone
U S 2 188 008	Jan 23 1940	S H Lassen Ass to F R Park Inc	Isolation of the vitamins of the B complex and especially of vitamin B ₁ from fish press water
U S 2 222 306	Nov 19 1940	A G Atwood	Simultaneous production of alcohol for bourbon and rye whiskies and a riboflavin containing material from grains

PATENT NO	DATE	PATENTEE	ABSTRACT
Isolation (Continued)			
U S 2 239 285	Apr 128 1941	G E Flanigan and G C Supplee	Isolation of vitamin B (riboflavin) from concentrates by precipitation of impurities in an acetone water solution with ethyl alcohol followed by ether extraction precipitation of foreign matter with acetone and final crystallization from the concentrated acetone solution
B 524 445	Aug 7 1940	N V Organon	Separation of riboflavin from its phosphoric acid ester by elution of an adsorbate of these substances with a dilute aqueous solution of an amine or acid amide or by electve adsorption of riboflavin from a dilute solution of an amine or acid amide
B 524 515	Aug 8 1940	N V Organon	Process for the separation of riboflavin from its phosphoric acid ester by distribution between water and a water immiscible solvent such as a phenyl substituted aliphatic alcohol or a mixture of a phenol with an aliphatic alcohol or a mixture of a phenol with a hydrocarbon
C 667 806	Nov 21 1938	K. Feudenberg Ass to I G	Elution of physiologically active adsorbates from metal sulfides by oxidation of the sulfides e g with H ₂ O ₂
Jap 110 826	May 17 1935	W N Kahra and B Inukai Ass to R Kenkyo Co	Concentrate of vitamin B by adsorption followed by enzymatic digestion of the adsorbate
Synthesis			
U S 2 155 555	April 1939	P Karrer Ass to Hoffmann LaRoche	Product elims for 7 methyl (or "alkyl") 9 (diethyl) iso-alloazine
U S 2 238 874 B 441 692 F 79 070	April 15 1941 Jan 20 1930 Dec 1 193	R Kuhn Ass to I G	Synthesis of vitamin B ₂ and of other iso-alloazine derivatives by condensation of alloxan with N-mono substituted aromatic o-diamine especially in acid solution and in the presence of boracic acid
U S 2,261 608	Nov 4 1941	M Tishler and J W Wellman Ass to Merck	Synthesis of riboflavin by reductive condensation of tetra acetyl ribonitrile with 4,5 dimethyl nitrile coupling with para nitro phenyl diazonium chloride and reduction to N tetra acetyl ribityl amino amino-4,5 dimethylbenzene followed by condensation with 5,5 dichlorobarbituric acid and final hydrolysis
B 457 984	Dec 10 1936	Hoffmann LaRoche	Process for the manufacture of iso-alloazine derivatives which consists in condensing derivatives of o-phenylenediamine which possess a hydroxylated aliphatic side chain attached to an amino group with alloxan

PATENT NO	DATE	PATENTEE	ABSTRACT
Synthesis (Continued)			
Swiss 187 938	Mar 1 1937	Hoffmann LaRoche	6 Methyl 9 (d ribityl) iso-alloxazine by condensation of 4 methyl 2 amino phenyl ribamine with alloxan
Intermediates for the Synthesis			
U S 2 152 602	April 4 1939	F P Phelps Ass to the Government of the United States	Manufacture of ribose by hydrolysis of nucleic acids
U S 2 159 804	May 23 1939	W E Lawson and C P Spaeth Ass to DuPont	N Ribityl 6 nitro 3 4 xylidine from ribitylamine and 6 nitro-3 4 dimethyl-chlorobenzene
U S 2 193 433	Mar 12 1940	P L Salzberg Ass to DuPont	Composition of matter claims for N ribityl 3 4 xylidine and process of preparation from 3 4 xylidine and ribose followed by hydrogenation
U S 2 237 074 B 457 178 G 677 515	April 1 1941 Nov 23 1938 June 27 1939	P Karrer Ass to Hoffmann LaRoche	1 Ribitylamino 2 amino 4 5 dimethyl benzene from N ribityl 3 4 xylidine by coupling with a diazonium salt followed by reduction of the azo dye
U S 2 250 999	July 29 1941	R Pasternack and J V Brown Ass to Pfizer	Composition of matter claims for tetra acetyl (3 4 dimethyl phenyl) ribityl amine and tetra acetyl (3 4 dimethyl 6 carbethoxy amino phenyl) d ribityl amine and process of making these compounds by condensation of tetra acetyl-d ribose with an aromatic amine followed by hydrogenation
B 461 245 G 642 148	Feb 8 1937 March 4 1937	R Kuhn and R Ströbele Ass to I G	Condensation of sugars with ortho-nitro-anilines followed by reduction
G 664 048 Continuation of 642 148	Aug 19 1938	R Kuhn and R Ströbele Ass to I G	Reduction of acyl-derivatives of condensation products of ortho-nitro-anilines and sugars followed by saponification
G 664 439 Continuation of 642 148	Sept 1 1938	R Kuhn and R Ströbele Ass to I G	Condensation of ortho-nitro-anilines with sugars in the presence of ammonium halides or amine hydrogen halides
G 679 001 Continuation of 642 148	July 27 1939	R Kuhn and R Ströbele Ass to I G	Reduction of the condensation products of ortho-nitro anilines and sugars in the presence of borates in slightly alkaline solution to the corresponding phenylene diamines
Derivatives and Utilization			
U S 2 068 623	Jan 19 1937	O Warburg Ass to Schering	Isolation of the yellow oxidation enzyme from yeast
U S 2 111 491 B 451 938 G 666 791 G 647 721 F 809 884	Mar 15 1938 Aug 10 1936 Oct 28 1938 Dec 7 1937 Mar 11 1937	R Kuhn F Weygand and H Rudy Ass to Winthrop	Preparation of 5 phosphoric acid ester of vitamin B

PATENT NO	DATE	PATENTEE	ABSTRACT
Derivatives and Utilization (Continued)			
U S 2 756 604	Sept 23 1941	E Aubagen	Water solutions of vitamin B ₂ using nicotinic acid in the form of its salts or <i>N</i> non substituted amide to increase the solubility
G 698 815	Nov 18 1940	Ass to Winthrop	
Continuation of G 686 793			
B 430 571	June 17 1935	O Warburg	Cleavage of the yellow oxidation ferment with sodium hydroxide at 50-60 C to produce lumilactoflavin
G 638 822	Nov 23 1936	Ass to Schering	
Continuation of G 638 138			
B 438 126	Nov 7 1935	G B Walden	Vitamin B is added to preparations for the treatment of pernicious anemia
Continuation of 411 179	June 7 1934	Ass to Eli Lilly	
B 451 779	Aug 11 1936	Schering	
G 637 503	Oct 29 1936		Preparation of the yellow oxidation ferment by precipitation in aqueous solution at pH 4 in the presence of an acetate buffer
F 785 490	Aug 10 1935		
B 495 718	Nov 18 1938	Ass to I G	
B 604 721	April 26 1939	J Eisenbrand and H Picher	Decomposition of vitamin B ₂ during sterilization is restrained by adding urea and an acid e.g. HCl to obtain a pH below 5 which pH after sterilization is between 5 and 7
G 672 018	June 22 1939	Ass to I G	
G 632 131	July 3 1936	R Kuhn and H Rud	
G 632 366	July 7 1936	Ass to I G	Manufacture of a diacetone vitamin B
G 633,399	July 25 1936	Schering	Riboflavin phosphoric acid ester from the yellow oxidation ferment from aqueous yeast extract
G 637 386	Oct 27 1936	H T Theorell	Riboflavin phosphoric acid ester from the yellow oxidation ferment by treatment with methanol
Continuation of 633 392		Ass to Schering	
G 638 138	Nov 10 1936	Schering	
Continuation of G 638 822			Purification of the riboflavin phosphoric acid ester by conversion into an alkali earth salt
G 686 793	Jan 16 1940	Schering	Cleavage of the yellow oxidation ferment with sodium hydroxide
G 687 197	Jan 24 1940	E Aubagen	Solution of vitamin B ₂ in mono- <i>V</i> alkyl derivatives of amides of lower fatty acids
		Ass to I G	
		Ciba	
G 688 047	Feb 10 1940		Preparation of vitamin B ₂ -phosphoric acid ester by phosphorylation of the vitamin by means of phosphatases from the mucous membranes of the intestines (in the presence of phosphates) and in the presence or absence of compounds of the adrenal cortex
Continuation of G 686 793		F Aubagen	Solutions of vitamin B ₂ in water using phenol or polyphenol sulfonic acid salts to increase the solubility
		Ass to I G	

VITAMIN B₅-PYRIDOXIN

PATENT NO	DATE	PATENTER	ABSTRACT
U S 2 248 078 B 534 916	July 8 1941 Mar 21 1941	S A Harris Ass to Merck	Synthesis of vitamin B ₅ by reacting ethoxyacetyl acetone and cyanoacetamide to form 3 cyano-4 ethoxy methyl 6 methyl pyridone 2 hydrolyzing to form the lactone of 3 carboxy 4 hydroxymethyl 6 methyl pyridone 2 treating with nitric acid chlorinating and reducing to form the lactone of 3 carboxy 4 hydroxy methyl 5 amino 6 methyl pyridine diazotizing to form the lactone of 3 carboxy 4 hydroxymethyl 5 hydroxy 6 methylpyridine and reducing the latter compound to form vitamin B ₅
U S 2 250 396 G 699 555	July 22 1941 Dec 2 1940	W Salzer Ass to Winthrop	Synthesis of vitamin B ₅ by degradation of 3 alkoxy quinaldine 4 carboxylic acid
U S 2 261 188	Nov 4 1941	J V Seudi Ass to Merck	Boric acid salts of vitamin B ₅
U S 2 266 754	Dec 23 1941	S A Harris Ass to Merck	Catalytic reduction of 3-cyano 4 ethoxy methyl 5 nitro 6 methyl pyridone 2 and acetylation of 3 cyano 4 ethoxy methyl 5 amino-6 methyl pyridone
U S 2 296 16	Sept 15 1942	Ass to I G	Fat soluble derivatives of vitamin B ₅ by acylation
B 534 917	Mar 21 1941	Ass to Merck	The vitamin B ₅ base from its hydrochloride
B 536 249	May 8 1941	Merck	2 Methyl 3 methoxy pyridine 4 5 dicarboxylic acid ester from the corresponding acid by esterification
B 538 000	July 16 1941	Merck	Lactones of 2 methyl 3 alkoxy 4 hydroxymethyl 5 carboxy pyridine by alkylating the lactone of 2 methyl 3 hydroxy 4 hydroxy methyl 5 carboxy pyridine
G 684 975	Dec 8 1939	R Kuhn and G Wenig Ass to I G	Isolation of a vitamin B ₅ protein symplex from animal or plant material
G 702 879	Feb 17 1941	K Westphal and H Andersag Ass to I G	Vitamin B ₅ by degradation of 3 methyl 4 alkoxy isoquinoline
G 702 830	Feb 17 1941	K Westphal Ass to I G	2 Methyl 3 methoxy 4 5 bis amino methyl pyrimidine from the corresponding 4 5 dinitrile by catalytic reduction
G 704 761	April 7 1941	K Westphal Ass to I G	Synthesis of vitamin B ₅ by the action of nitrous acid on 4 5 bis amino methyl methoxy pyridine

VITAMIN B₅

U S 1 976 175 B 396 135	Oct 9 1934 Aug 3 1933	C L Lautenschläger and F Lindner Ass to Winthrop	Production of muscle adenosine phosphoric acid from yeast adenosine triphosphoric acid by hydrolysis
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PATENT NO	DATE	PATENTEE	ABSTRACT
U S 098 976	Nov 16 1937	S L Ruskin Ass to F R Ruskin	Solutions or tablets for pharmaceutical uses comprising isotonic solutions of compounds of an isolated nucleotide such as adenylic acid and Hg Ca Fe Au Ag or Al
B 396 647	Aug 10 1933	I G	Alkali metal salt of adenyli pyrophosphoric acid
B 413 430	July 19 1934	I G	Muscle adenylic acid from muscle adenyli pyrophosphoric acid by hydrolysis with an alkali earth metal hydroxide
B 426 866 G 638 418	April 10 1935 Nov 14 1936	Ciba	Nucleotides of adenylic acid are acylated
G 591 926	Jan 29 1934	C L Lautenschläger and F Lindner Ass to I G	Isolation of adenylic phosphoric acid by hydrolysis of yeast extracts containing adenosine triphosphoric acid

NICOTINIC ACID

U S 1403 117 B 184 655 G 351 085	Jan 10 1922 Sept 14 1922 April 3 1922	M Hartmann and M Seiberth Ass to Ciba	Product claims for dialkyl amides as medicinals. Process claims reaction of nicotinic acid halide and a salt of a dialkylamine
U S 1611 978	Dec 28 1926	R Wolfenstien	Terpene alcohol esters of nicotinic acid
U S 1617 332 G 441 707	Feb 15 1927 Mar 10 1927	M Hartmann and M Seiberth Ass to Ciba	Manufacture of pyridine-3-carboxylic acid amides substituted in the amide group by reaction of a quolinic acid anhydride with a secondary amine followed by decarboxylation
U S 1938 753	Dec 5 1933	M Hartmann and M Seiberth Ass to Ciba	Addition product of nicotinic acid and its derivatives with water soluble salts of alkali earth metals
U S 2 136 501	Nov 15 1938	M Hartmann and L Panizzone Ass to Ciba	Mercuric N-propyl nicotin amides
U S 2 136 503 Div of U S 2 136 501	Nov 15 1938	M Hartmann and L Panizzone Ass to Ciba	N-substituted nicotinamide mercuric compounds
U S 2 141 128	Dec 20 1938	H v Euler H Albers and F Schlenk Ass to E Bihler	Purification of coenzyme I by precipitation with barium hydroxide and fraction adsorption on alumina. Also by precipitation with a monovalent copper halide followed by precipitation with barium hydroxide
U S 2 146 899	Feb 14 1939	F Karrer Ass to Hoffmann LaRoche	Manufacture of 3-carboxylic acid amides of acetylated N-glycoside pyridinium bromide

PATENT NO	DATE	PATENTEE	ABSTRACT
U S 2 188 244	Jan 23 1940	G A Langlois and A M De	Morpholine amide of nicotinic acid
B 503 780	April 12 1939	loison	
F 824 042	Jan 31 1938	Ass to Fabriques de Produits	
F 824 042/49 160	Nov 28 1938	de Chimie Organique de Loire	
(1st add)			
F 824 042/49 840	Aug 8 1939		
(2nd add)			
U S 2 233 419	Mar 4 1941	E E Moore	Composition of matter claims for
		Ass to Abbott	
B 254 747	Dec 20 1914	Ciba	straight chain aliphatic amine salt
B 430 051	July 9 1936	K Fricker	of nicotinic acid for parenteral in
C 653 873	Nov 18 1937		jection
F 791 783	Dec 17 1934		Dialkylamides of nicotinic acid
G 529 319	July 11 1931	Knoll A G	Synthesis of N substituted amides
G 544 389	Mar 12 1931	Ciba	of pyridine-carboxylic acids by con
G 554 008	July 4 1932	Knoll A G	densation of the acid with second
Continuation of			ary aliphatic or cyclic amines in the
G 529 319			presence of phosphorous pentoxide
G 603 733	Oct 6 1934	Ciba	Purification of cozymase by pre
G 654 960	Jan 8 1938	O Warburg and W Christian	cipitation with picric acid followed
G 667 696	Mar 14 1939	Ass to Schering	by decomposition with silicotung
G 668 388	Mar 22 1939	H Albert and F Schlenk	stic acid
F 849 519	Nov 25 1939	Ass to I G	Double compound of nicotinic acid
Hung 110 083	June 1 1934	Chinoin Gyógyszer es Veg	with salts such as CaCl_2 MgCl_2 etc
Russ 35 836	April 30 1934	yzseti	Purification of cozymase by precipi
Russ 44 504	Oct 31 1934	I L Knunyantz and M M	tation with aluminum sulfate and
		Katznel son	decomposition by means of phos
		Y L Goédfarb	phoric acid The cozymase ob
			tained is further purified according
			to G 529 319
			Urethanes of nicotinic acid
			Isolation of codehydrogenase II
			Cozymase
			Enzymatic synthesis of muscle
			adenylic acid and of cozymase from
			adenosine
			Free nicotinic acid is heated with
			substituted carbamyl chlorides
			Dialkyl amino ethyl esters of neo
			tic acid
			Preparation of 2 naphthyl nicotin
			ate

PANTOTHENIC ACID

U S 2 234 680	Mar 11 1941	M B Moore	Synthesis of pantothenic acid from
		Ass to Abbott	
U S 2 271 872	Feb 3 1942	H K Mitchell	Production of pantothenic acid
		Ass to Research Corp	

beta alanine and alpha gamma
dihydroxy beta beta dimethyl
butyric acid or alpha hydroxy beta
beta dimethyl gamma butyrolactone

analogues having in the beta position of the butyric acid part one or two methylol groups

PATENT NO	DATE	PATENTER	ABSTRACT
B 535 988	April 29 1941	Merck	Synthesis of pantothenic acid by condensation of beta alanine or a salt or ester thereof with alpha hydroxy beta beta dimethyl gamma butyrolactone or the corresponding hydroxy or keto-acid

INOSITOL

U S 1 072 989 (Also Swiss 62 459)	Sept 9 1913 June 5 1912	E Preisswerk Ass to Ciba)	Iron salts of inositol phosphoric acid absorbed on albumin
U S 1 721 214 B 308 403	July 16 1929 Jan 24 1928	F Goedeck	Dietary composition containing vitamins and inositol he a phosphate and its Ca salts from the same plant source
U S 2 112 553	Mar 29 1938	E Bartow and W W Walker	Inositol from phytin by decomposition with Ca(OH) ₂
B 216 982	Mar 28 1923	G Bruns	Methoxyisobutyl pentaphosphate
C 684 946	Dec 8 1939	F Fischler	Isolation of alkaline earth salts of inositol tetraphosphate
Swiss 91 727	Nov 16 1921	S Pasternak	Inositolpolyphosphate by phosphorylation of inositol
Swiss 141,002	Dec 8 1928	Crustallo A G	Extraction of Ca inositol he a phosphate and vitamin from vegetable matter

VITAMIN C

Isolation			
U S 1 468 731 B 193 831 C 417 020 F Add 26 034 and 77 271 to F 532 398	Sept 18 1923 May 6 1924 Aug 8 1923	L A Agopian	Extraction of vitamins from fresh plant juices to which organic acids have been added
U S 1 745 788	Feb 4 1930	C Funk	Antiscorbutic vitamin from lemon juice by precipitation as lead salt
U S 078,237	April 27 1937	O Dalmer and H Wieters Ass to Merck	Isolation of ascorbic acid from gladiolus leaves
U S 2 233 417	Mar 4 1941	C G King and W A Waugh	Isolation of vitamin C from lemon juice
B 133 183	Oct 9 1919	Ciba	Vitamin C preparations from vegetable materials by adding acids as stabilizers and concentrating the mixture
B 168 903 B 265 655 G 415,313 F 53 398	Mar 6 1923 April 7 1927 June 19 1935 Feb 2 1922	L A Agopian	Preparation of vitamins from fresh plant juices by precipitation with salts of heavy metals and by evaporation in the absence of air extraction of the vitamins with suitable solvents.
B 772 376 F 695 537	June 16 1927 Oct 5 1923	L A Agopian	Pure vitamin C from plant juices by precipitation with lead salts and recrystallization from alcohol and acetone

PATENT No	DATE	PATENTER	ABSTRACT
<i>Isolation (Continued)</i>			
G 397 886	June 27 1924	G Eichelbaum	Antiscorbutic calcium preparation from fruit or plant juices by the addition of calcium hydroxide
G 637 258	Oct 27 1936	K L Lautenschläger and F Lindner Ass to I G	Isolation of ascorbic acid from leaves of plants of the Polygonaceae family
G 661 176	June 14 1938	K L Lautenschläger and F Lindner Ass to I G	Improved method of isolating ascorbic acid by soaking plant material in solutions of strong acids and further processing according to known methods
F 850 539	Dec 19 1939	Établissements Byla	Vitamin C concentrates are purified by dialysis in acid solution
F 850 981	Mar 7 1940	Établissements Byla	Vitamin C extraction from agaves
Hung 108 184	Feb 1 1934	Ass to Chinoin Gyógyszer	Purification of vitamin C-containing aqueous plant extracts by precipitation with lead and barium salts
Russ 48 318	Aug 31 1936	A A Schmidt and K S Tutschinskaja	Concentrate of ascorbic acid from hips
<i>Synthesis</i>			
U S 2 036 126	Sept 29 1936	T Reichstein	l Ascorbic acid from l xylosone by condensation with hydrocyanic acid followed by hydrolysis
B 425 198	Mar 8 1935	Ass to Hoffmann LaRoche	
G 624 509	Feb 11 1936		
F 770 816	Sept 21 1934		
U S 2 068 453	Jan 19 1937	B Helferich and O Peters	Vitamin C by condensation of glyoxylic acid ester with l threose
B 460 586	Feb 1 1937		
G 637 448	Oct 29 1936		
U S 2 073 707	Mar 9 1937	W N Haworth E L Hirst	Ascorbic acid is prepared by oxidizing l sorbose with nitric acid to 2 keto l gulonic acid and enolizing
B 443 901	Mar 2 1936	J K N Jones and F Smith	
F 794 221	Feb 11 1936	Ass to British Drug Houses	
U S 2 129 317	Sept 6 1938	F Elger Ass to Hoffman LaRoche	Ascorbic acid by acid treatment of 2 keto l gulonic acid its ester or diacetone compound
U S 2 159 191	May 23 1939	W Wenner	Ascorbic acid from bis methylene ethers of 2 keto l gulonic acid or diacetone 2 keto-l gulonic acid by means of acids at elevated temperatures
B 461 790	Feb 24 1937	Ass to Hoffmann LaRoche	
G 641 639	Feb 13 1937		
F 806 976	Dec 29 1936		
U S 2 160 021	May 30 1939	H Obie	Ascorbic acid from 2 keto gulonic acid by basic agents
B 420 264	June 17 1935		
G 648 311	Aug 20 1937		
U S 2 165 151	July 4 1939	R Pasternack and P P Regna	l Ascorbic acid from esters of 2 keto l gulonic acid by treatment with a metal e g iron nickel cobalt manganese cadmium and zinc in aqueous solution
B 521 831	May 31 1940	Ass to Pfizer	
U S 2 165 184	July 4 1939	R Pasternack and P P Regna Ass to Pfizer	l Ascorbic acid from esters of 2 keto l gulonic acid by treatment with magnesium in water and alcohol or dioxane solution
U S 2 179 977	Nov 14 1939	F Elger Ass to Hoffmann LaRoche	l Ascorbic acid from 2 keto-l gulonic acid or derivatives thereof by acid treatment in a mixture of alcohol and chloroform

PATENT NO	DATE	PATENTEE	ABSTRACT
<i>Synthesis (Continued)</i>			
U S 2 179 978 G 104 760 G 673 480	Nov 14 1939 April 7 1941 Mar 2 1939	F Eiger Ass to Hoffmann LaRoche	l Ascorbic acid from 2 keto-l gulonic acid or its alkyl esters by heating in the presence of an alkali salt of a weak acid in anhydrous alcohol
U S 2 18 383 B 516 115	Jan 2 1940 Dec 22 1939	R Pasternack and G O Cragwall Ass to Pfizer	l Ascorbic acid from 2 keto l gulonic acid by the action of glacial acetic acid and a mineral acid
U S 2 189 830 G 676 011	Feb 13 1940 May 24 1939	O Zima Ass to Merck	l Ascorbic acid from diacetone 2 keto gulonic acid by acid treatment
U S 2 189 778	Feb 13 1940	O Dalmer and K Heyns Ass to Merck	l Ascorbic acid from sorbose by catalytic oxidation followed by acid treatment
U S 2 190 167 G 684 72	Feb 13 1940 Dec 4 1939	O Zima Ass to Merck	l Ascorbic acid from methylene ether derivatives or dibenzal derivatives of 2 keto l gulonic acid by treatment with concentrated HCl
U S 2 200 374	July 2 1940	I Stone Ass to Wallerstein Co	Ascorbic acid from ozones plus hydrocyanic acid the ozone being prepared by oxidation of sugars with cupric ion in aqueous organic acid solution
U S 207 090 C 683 924	July 2 1940 Nov 18 1939	H Helferich Ass to Winthrop	Ascorbic acid by condensation of mesoallic acid ester with l threose
U S 20 111	Dec 2 1941	T Reichstein Ass to Hoffmann LaRoche	Ascorbic acid from l sorbose + 2 keto-l gulonic acid or methyl ether derivative thereof
B 478 814 C 673 485 F 779 883	May 20 1935 Mar 28 1939 April 13 1930	T Reichstein Ass to Hoffmann L Roche	Ascorbic acid from 2 keto-l gulonic acid esters by alkali saponification followed by lactonization and acid solution
B 4 8810	May 20 1933	T Reichstein	Ascorbic acid from 2 keto-l gulonic acid by acid treatment
B 4 9207	Jan 4 1937	T Reichstein	Ascorbic acid from 2 keto l gulonic acid or its derivatives which readily split with acids by acids in alcoholic solution
B 401 48	May 31 1937	T Reichstein	Ascorbic acid by heating 2 keto gulonic acid or its derivatives which are easily split with acids
B 409 17	July 20 1937	T Reichstein	Ascorbic acid from 2 keto gulonic acid by treating with alkali salts of weak acids and converting the so-formed alkali salts of ascorbic acid by means of strong acids into ascorbic acid
C 670 4 Cont Int n of C 6348	June 1 1941	Hoffmann LaRoche	Ascorbic acid from 2 keto-l gulonic acid or derivatives which are hydrolyzed easily by acids by treatment with acids in the presence of a solvent in which the formed ascorbic acid is insoluble

PATENT NO	DATE	PATENTEE	ABSTRACT
Intermediates for the Synthesis			
U S 2 140 480	Dec 13 1938	T Reichstein	2 keto <i>l</i> gulonic acid from sorbose via a bis methylene ether derivative or acetals of cyclic ketones by oxidation with permanganate
U S 2 039 929	May 6 1936	Ass to Hoffmann LaRoche	
B 428 815	May 20 1935		
B 477 786	April 18 1935		
B 435 971	Oct 2 1935		
G 699 877	Nov 7 1940		
G 703 297	Mar 4 1941		
F 780 050	April 18 1935		
F 45 774	Dec 3 1935	-	
U S 2 103 311	April 4 1939	R Pasternack and P P Regna Ass to Pfizer	Oxidation of <i>l</i> gulonic acid to 2 keto <i>l</i> gulonic acid by chromic acid
U S 2 157 137	April 9 1939	R T Major and E W Cook Ass to Merck	Xyloseen tribenzoates and process for producing the same
U S 2 188 777	Jan 30 1940	R Pasternack and P P Regna Ass to Pfizer	Oxidation of soluble aldonates to 2 keto aldonates by means of chlorates in the presence of a vanadium catalyst
B 534 746	Mar 17 1941		
U S 2 190 377	Feb 13 1940	O Dalmer and K Heyns	2 Keto <i>l</i> gulonic acid from sorbose by catalytic oxidation at pH of about 6 to 11
B 490 050	Nov 7 1938	Ass to Merck	
F 829 236	June 16 1938		
U S 2 194 476	Mar 26 1940	R T Major and E W Cook Ass to Merck	Nitriles of fully acetylated 2 keto sugar acids and process for their production
See U S 2 198 628	April 30 1940		
U S 2 198 628	April 30 1940	R T Major and E W Cook Ass to Merck	Fully acetylated sugar acid chlorides To be used in U S 2 194 476
U S 2 207 991	July 16 1940	R Pasternack and I P Regna Ass to Pfizer	2 Keto aldonic acid esters from aldonic acids by chlorates in the presence of a vanadium catalyst and a mineral acid using non aqueous lower alcohols as solvents
U S 2 222 155	Nov 11 1940	R Pasternack and P P Regna Ass to Pfizer	2 Keto aldonic acids by subjecting an aldinate to anodic oxidation in a weakly acid aqueous solution containing a soluble chromium compound and a member selected from the group consisting of alkali metal and alkaline earth metal chlorides and bromides
G 544 066	Feb 20 1937	J Muller and U Hoffman Ass to I G	Manufacture of polyvalent alcohols by catalytic reduction of sugars with hydrogen
G 627 243	Mar 23 1936	Hoffmann LaRoche	1 Xylose from <i>d</i> sorbitol acetate by oxidation
G 644 060	May 19 1937	Hoffmann LaRoche	2 Keto- <i>l</i> gulonic acid from <i>l</i> sorbose by direct oxidation with H ₂ O
I 851 347	Jan 6 1940	A Corbellini	The acetone condensation product of a 2 keto laevo gulonic acid is heated with methanol and sulfuric acid to form methyl ethers with the hydroxyl groups of the acid. These methyl ethers are of value in the synthesis of laevo-ascorbic acid (vitamin C)

PATENT NO	DATE	PATENTER	ABSTRACT
<i>Intermediates for the Synthesis</i> (Continued)			
Swiss 175 347	May 1 1935	T Reichstein	Preparation of the methyl ester of keto-hexonic acid from the free acid with HCl or H ₂ SO ₄ in methanol
<i>Derivatives and Utilization</i>			
U S 1 886 931	Nov 2 1932	E R Alexander Ass to the Vitamin Corp	Process of preserving a concentrated vitamin extract by incorporation into a vitamin preserving acidic fruit mass
U S 2 035 153	Mar 24 1936	F Elger	Ascorbic acid as stabilizer for neo-salvarsan
B 439 93,	Dec 17 1935	Ass to Hoffmann LaRoche	
G 660 703	June 1 1938		
U S 2 058 270	Oct 20 1936	I Elger	Addition of 2 keto-L gulonic acid esters to foods
G 6 97 3	May 9 1936	Ass to Hoffmann LaRoche	
F 788 014	Oct 10 1931		
U S 2 117 777	May 17 1938	K Warnat	Double salt of calcium ascorbate and of calcium salts of polyhydroxy mono-carboxylic acids
G 70 185	Feb 4 1941	Ass to Hoffmann LaRoche	
U S 2 132 66	Oct 11 1938	I H Volwiler and M B Moore	Aliphatic amine salts of ascorbic acid and process for producing them
U S 134 746	Oct 25 1938	F Elger	Ascorbic acid salts of histidine
R 480 503	Feb 23 1938	Ass to Hoffmann LaRoche	
U S 2 140 989	Dec 20 1938	J Eisenbrand and M Lenz	Molecular compounds from ascorbic acid or isoascorbic acid and quinine or quinidine
B 499 798	Jan 30 1939	Ass to Winthrop	
B 499 840	Jan 30 1939		
U S 2 150 140	Mar 7 1939	K Warnat	3,6-Tribenzoyl ascorbic acid and its preparation from ascorbic acid salts and benzoyl chloride
U S 2 159 214	May 23 1939	S Klein	Double salt of calcium ascorbate and calcium cetylascitylate
U S 2 159 986	May 30 1939	P P Gray and I Stine	Addition of ascorbic acid to aqueous emulsions
U S 2 187 467	Jan 16 1940	E H Stuart	Aqueous solution of a salt of ascorbic acid e.g. of alkali metal alkali earth metal ammonium the lower alkyl substituted amines and containing sulfur compounds e.g. sodium hydroxide
U S 2 192 831	Aug 27 1940	F Hoffmann and P M Equardt	Manufacture of stable derivatives of adrenaline by reacting adrenaline with ascorbic acid
U S 2 213 977	Sept 10 1940	W G Christiansen	Vitamin C amine solution stabilized by means of hypophosphite
U S 2 237 099	Feb 2 1941	U H Engels, J Wejland and R T Schenck	Stabilization of ascorbic acid solutions by water soluble edible colloids of the group consisting of albumen, casein, raw milk, gelatin and starch
U S 2 232 712	Feb 20 1941	R T Major and E W Cook	Process for the production of fully acetylated sugar acids from corresponding aldehyde-sugar acetates and corresponding lactones
		Ass to Merck	Composition of matter claims for fully acetylated sugar acids which do not contain a keto group

PATENT NO	DATE	PATENTER	ABSTRACT
Derivatives and Utilization (Continued)			
U S 2 249 903 G 693 375	July 22 1941 July 6 1940	C L Lautenschläger and F Lindner Ass to I G	Preparation of stable water solu- tions of ascorbic acid by partial or complete neutralization with or ganic bases
U S 2 251 526 B 524 319	Aug 5 1941 Aug 2 1940	A Salomon Ass to N V Orgachemia	Stable concentrated quinine solu- tions comprising dissolving a mono salt of quinine with the acid of as- corbic acid
U S 2 254 483	Sept 9 1941	G F D Alelio Ass to General Electric	Addition of ascorbic acid as an in- hibitor to polymerizable materials containing a methylene group
U S 2 260 870	Oct 28 1941	S I Ruskin	Manganese compound of a corbic acid
B 455 721 F 797 675	Oct 28 1936 July 1 1936	Dansk Gaering Industri	Addition of ascorbic acid to flour and dough
B 469 335	July 23 1937	I G	Stable solutions of partly or com- pletely neutralized ascorbic acid for ampules
B 472 531 F 817 578	Sept 20 1937 Sept 6 1937	Ciba	Process for the manufacture of fer- rous salts of ascorbic acid
B 485 617 F 823 982	May 23 1938 July 18 1938	H Lotze	Addition of catalase to preparations containing vitamins A B C and/or D for protecting vitamin C against destruction
B 486 055	May 20 1938	H W Rhodehamel and E C Kleiderer Ass to Eli Lilly	Anhydrous solution of vitamin C in propanol
B 486 546	June 7 1938	P A Henriksen Ass to Canned Cream & Milk Co	Addition of vitamin C to milk or liquid milk products
B 486 757 G 687 875	June 9 1938 Oct 24 1939	F B Dehn Ass to Promonta	Preparation of ferrous salts of as- corbic acid
B 488 784	July 13 1938	S L Ruskin	Process for the production of the following salts of a corbic acid alkali calcium barium strontium iron manganese bismuth arsenic gold silver copper mercury zinc aluminum and tin
B 495 675	Nov 17 1938	Hoffmann LaRoche	Process for the manufacture of cal- cium double salts of ascorbic acid and poly oxy mono carboxylic acids
B 503 476	Mar 30 1939	N V Industrielle Maatscha- prij voorheen Noutry & van der Lande	Mixture of ascorbic acid with cal- cium phosphates gypsum silica talc magnesium carbonate potas- sium sulfate wheat rice maize or potato starch
B 509 709	July 19 1939	N V Orgachemia	Stabilization of ergometrine by as- corbic acid
B 511 585	Aug 21 1939	Ocean	Addition of vitamin C containing paprika to foodstuffs
B 514 047	Oct 30 1939	L E Wells and Rountree Co	Particles of ascorbic acid coated with gelatin gum or hard sugar

PATENT NO	DATE	PATENTEE	ABSTRACT
Derivatives and Utilization (Continued)			
B 517 348 F 829 547	Jan 26 1940 June 29 1938	P R A Maltha	Addition of ascorbic acid to form in a mixture with metals or salts of copper manganese cobalt iron salts proteic acid salts or proteins containing such metals or powder of squash cucumbers cabbage leaves or tomatoes
B 533 480	Feb 13 1941	Hoffmann LaRoche	Ascorbic acid salts of cinchona alkaloids
G 470 019	Jan 3 1939	J Korselt	Preparation of pressed plant juices rich in vitamins from chlorophyll containing plants by the addition of calcium salts and aliphatic oxyacids
C 630 776 (See continuation 701 561)	Dec 19 1938	Hoffmann LaRoche	Preparation of esters of ascorbic acid and higher fatty acids
G 663 987	Aug 18 1938	Merek	Dissolution of monoalkali or alkaline earth salts of ascorbic acid for filling ampoules followed by sterilization
G 681 859	Oct 3 1939	Madaus & Co	Preparation of stable colloidal solutions of phosphorus in alcohol by the addition of ascorbic acid
G 696 794	Sept 30 1940	Madaus & Co	Stabilization of colloidal bismuth containing aqueous solutions for injections by adding ascorbic acid in the presence of protective colloids
G 699 327 Continuation of G 693 375	Nov 7 1940	C L Lautenschläger and F Lindner Assign to I G	Preparation of stable water solutions of ascorbic acid by partial neutralization with inorganic bases
G 701 561 Continuation of G 639 776	Jan 18 1941	Hoffmann LaRoche	Esters of ascorbic acid by reaction of salts of ascorbic acid with acid halides of aromatic carboxylic acids or by reaction of ascorbic acid with acid halides in the presence of organic bases
F 749 74	July 28 1933	Carl Colln & Strimann	Addition of sterilized plants containing vitamin C to evaporated milk
F 800 599	July 8 1936	Société Française Des Sucres	Addition of solutions of vitamins C and D to sugar or sugar syrup
F 803 335	Nov 17 1938	Erleong A Morel and A Josserand	Preparation of complex salts of ascorbic acid
F 805 335/47 105	Feb 6 1937	Erleong A Morel and A Josserand	Preparation of ammonium or amine-containing complex salts of ascorbic acid
F 807 877	Jan 23 1937	Erleong A Morel and A Josserand	Preparation of complex salt of ascorbic acid
I 8 3739	Jan 20 1938	Erleong A Morel and A Josserand	Preparation of complex salts of iso vitamin C
F 877 149	April 20 1938	N V Industriële Maatschappij Vorheen Noutry & van der Land	Preservation of food by the addition of ascorbic acid together with non hygroscopic substance

PATENT No	DATE	PATENTEE	ABSTRACT
Provitamins D (Continued)			
B 504 051	April 19 1939	The International Yeast Co and W G Bennett	Extraction of ergosterol from yeast by plasmolysis or boiling with acid followed by extraction with an organic fat solvent and saponification
G 542 667	Jan 28 1932	Merck	Ergosterol from yeast residues
G 553 910	July 2 1932	Hoffmann LaRoche	Isolation of sterols from yeast by heating yeast with aqueous alkali solutions in an open vessel
Austrian 140 190	Jan 10 1935	W Halden	Cultivation of yeast high in ergosterol under aerobic conditions
Belg 416 160	June 19 1936	N V Philips Gloeilampen fabrieken	Purification of ergosterol by esterification followed by absorption
Swiss 129 879	Jan 2 1939	Ciba	Isolation of ergosterol from fungi by alkali saponification
Swiss 201 169	Feb 1 1939	I G	Preparation of 7 dehydro cholesterol from 7 oxo cholesterol by reduction to 7 hydroxy cholesterol diacylation heat decomposition to a 7 dehydro cholesterol ester and final saponification
Conversion of Provitamins D to Vitamins D			
U S 1 680 818	Aug 14 1928	H Steenbock	Antirachitic activation of foods by ultraviolet irradiation
B 236 197	June 30 1924	Ass to Wisconsin Alumni Research Foundation	
F 587 187	April 14 1935		
U S 1 681 120	Aug 14 1928	A J Pacini Ass to M Richter	Production of antirachitic material from growth producing substances by irradiation with light of wave lengths longer than $30.2 \times 10^8 \text{ \AA}$
U S 1 682 318	Aug 28 1928	F C Beardslee Ass to F C Beardslee B M Huffine and J I Huffine	Apparatus for ultraviolet irradiation of food materials
U S 1 704 173	Mar 5 1929	J W D Chesney Ass to Solar Research Corp	Method of making the ultraviolet rays of the sun available for the production of the antirachitic principle in substances susceptible to such activation by separating solar ultraviolet rays of wave lengths within the range of substantially 2900 to 3700 Angstrom Units from the remaining rays and intensifying the separated ultraviolet rays by concentration
U S 1 723 603	Aug 6 1929	J W D Chesney Ass to Chesney Process Inc	Process for activating pasteurized liquids by addition of an organic acid followed by ultraviolet irradiation
U S 1 754 434	April 15 1930	J Perino	Vegetable alimentary materials are subjected in the absence of oxygen and in the presence of soluble phosphates to an irradiation by ultraviolet rays and are heated to not above 60°C during the irradiation
U S 1 762 105	June 3 1930	A J Pacini	Vitamin D by irradiation of sterols or fats with infra red or ultraviolet light in inert gas.
B 356 793	June 6 1930	Ass to C M Richter	

PATENT NO	DATE	PATENTEE	ABSTRACT
Conversion of Provitamins D to Vitamins D (Continued)			
U S 1 771 343	July 22 1930	A J Pacini Ass to C M Richter	Production of vitamin D by irradiation of sterols in the presence of a suitable photocatalyst such as halogen or by rays longer than 3022 Å or by electromagnetic radiation in the presence of a catalyst
U S 1 796 134	Mar 10 1931	A Wörner and F Kielwein	Claims a baking oven containing a mercury vapor lamp and a reflector for indirect irradiation and also claims irradiation of yeast therein
G 564 401	Nov 18 1932		
G 608 77	Jan 19 1935		
G 647 5 2	July 6 1937		
U S 1 808 760	June 9 1931	C E Bills Ass to Mead Johnson	Apparatus for irradiating fluids such as oils mixed with ergosterol
U S 1 817 938	Aug 11 1931	G C Supplee Ass to The Borden Co	Process of irradiating milk with ultraviolet rays
U S 1 842 313	Jan 19 1932	N K Chaney Ass to National Carbon Co	Apparatus for irradiating foods with ultraviolet rays
U S 1 848 305	Mar 8 1932	C E Bills Ass to Mead Johnson	Method of irradiating a stream of fluids
U S 1 871 135	Aug 9 1932	H Steenbock Ass to Wisconsin Alumni Research Foundation	Preparation of antirachitic food substances by ultraviolet irradiation of cereals
U S 1 871 136	Aug 9 1932	H Steenbock	Antirachitic product by ultraviolet irradiation of lipoids
B 314 94	Aug 28 1929	Ass to Wisconsin Alumni Research Foundation	
G 605 960	Nov 29 1934		
U S 1 873 942	Aug 23 1932	A Windaus	Antirachitic preparations by irradiation of ergosterol solutions until digitonin yields only a small precipitate
B 293 557	Not accepted	(In Great Britain—E Merck)	
U S 1 880 977	Oct 4 1932	A J Pacini Ass to Sun A-Sured Inc	Production of vitamin D by heating lipoids such as cholesterol with a photocatalyst such as uranium acetate
U S 1 880 978	Oct 4 1932	A J Pacini Ass to Sun A-Sured Inc	Process of producing vitamin D by heat extracting ergosterol from natural sources in the presence of a photocatalyst
U S 1 894 158	Jan 10 1933	N K Chaney Ass to National Carbon Co	Antirachitic activation of food stuffs by irradiation from an arc
U S 1 898 191	Feb 7 1933	W Zimmermann and W Frankenburger	Production of vitamin D from ergosterol by ultraviolet rays and interrupting the exposure before the maximum absorption in the range of the spectrum between $\lambda = 300m\mu$ and $\lambda = 230m\mu$ has been reached
B 796 093	Aug 26 1927	Ass to Wuthrop	
B 316 803	Aug 29 1929		
G 499 524	June 7 1930		
F 659 443	Aug 24 1928		
U S 1 904 751	April 18 1933	B H Reerink and A van Wijk	Irradiation of ergosterol with wave lengths from 270 to 300 $m\mu$
B 343 5 8	Mar 19 1931	Ass to N V Philips Gloeilampenfabrieken	
G 634 146	Aug 18 1936		
F 700 312	Feb 27 1931		
F 40 142	Apr 120 1932		
U S 1 920 587	Aug 1 1933	A J Pacini Ass to Sun A-Sured Inc	Method of producing vitamin D comprising suspending lipoids such as cholesterol and treating them with aspergillus oryzae

PATENT NO	DATE	PATENTER	ABSTRACT
Conversion of Provitamins D to Vitamins D (Continued)			
U S 1 928 397	Sept 26 1933	E D Shumway Ass to Quaker Oats	Production of an antirachitically activated cereal by removing the husk or skin from cereal and subjecting the whole kernel to the action of activating rays
U S 1 934 063	April 10 1934	J H Bragg	Apparatus for increasing the vitamin content of liquid food comprising a food chamber having openings for the passage of liquid food a source of ultraviolet rays and means to effect a cooling of the contents of the chamber
U S 1 935 054 B 394 408	April 17 1934 June 29 1933	R F Light and C N Frey Ass to Standard Brands	Process of activating antirachitically activatable unsaponifiable lipoids in dioxane by light containing frequencies below the visible spectrum
U S 1 966 546 U S 1 966 547	July 17 1934 July 17 1934	G P Goode Ass to General Development Lab	Apparatus for irradiating solutions of ergosterol etc with filtered ultraviolet light
U S 1 980 971	Nov 13 1934	H G Campsie	Production of vitamin D from provitamin D by means of radiant energy of 2536 to 2540 Angstrom Units
U S 1 982 078	Nov 27 1934	G Sperti Ass to General Development Laboratories	Process of increasing the vitamin content of food substances by subjecting them to soft x rays having wave lengths between approximately 2 and 13 Angstrom Units
U S 1 982 020	Nov 27 1934	G Sperti R J Norris R B Withrow and H Schneider Ass to General Development Laboratories	Process for treating food substances with ultraviolet light
U S 1 983 944	Dec 11 1934	A J Pacini Ass to American Research Products	Process of activating provitamin D by treatment with cathode rays in the presence of a suitable catalyst such as chlorine or bromine
U S 2 007 765 B 297 926	July 9 1935 Aug 22 1939	A Knudson Ass to Sun A Sured Inc	Process of increasing the vitamin D content of food fats or ergosterol by treatment with high velocity electrons
U S 2 013 273 U S 2 013 263	Sept 24 1935 Sept 24 1935	R M Fraps	Apparatus for irradiating materials such as ergosterol by sunlight
U S 2 015 282	Sept 24 1935	A J Pacini Ass to American Research Products Inc	Production of vitamin D from provitamin D by radioactive substances
U S 2 037 393	Oct 13 1936	H Steenbock Ass to Wisconsin Alumni Research Foundation	Yeast is antirachitically activated by treatment with light rich in ultraviolet rays
U S 2 104 681	Jan 4 1938	G C Supplee Ass to The Borden Co and to National Carbon Co	Method of irradiating liquid milk products with ultraviolet energy to give increased antirachitic potency by impinging the ultraviolet energy in such a manner that all rays of energy impinge obliquely upon the surface

PATENT NO	DATE	PATENTEE	ABSTRACT
Conversion of Provitamins D to Vitamins D (Continued)			
U S 2 106 773	Feb 1 1938	C C Whittier	Production of vitamin D by passing a vaporized provitamin D through a zone of electrically induced antirachitically activating discharge in a vacuum containing sodium emanations
U S 2 106 780	Feb 1 1938	C C Whittier	Method of producing vitamin D which consists in vaporizing ergosterol passing the same through a zone of electrical discharge in a vacuum tube condensing the treated ergosterol vapor and subjecting the condensate to a transversely directed electrical discharge in the vacuum tube
U S 2 106 781	Feb 1 1938	C C Whittier Ass to Nutrition Research Laboratories	Apparatus for the activation of provitamin D by the electrical discharge method
U S 2 106 782	Feb 1 1938	C C Whittier Ass to Nutrition Research Laboratories	Apparatus for the activation of provitamin D by the electrical discharge method
U S 2 11 242	Mar 29 1938	B Kramer and A E Sobel	Process for the antirachitic activation of provitamins D by an electrical discharge method
U S 2 117 100	May 10 1938	N A Mills Ass to DuPont	Preparation of antirachitic substances from provitamins D by the action of a high frequency electrical oscillating discharge
U S 2 128 199 C 642 759	Aug 23 1938 Mar 16 1937	A Windaus Ass to Winthrop	Preparation of an antirachitically active substance by ultraviolet irradiation of 2'-3-dihydro ergosterol
U S 2 151 645	Mar 21 1939	H C Stephens and S B Hoar Ass to Natural Food Products	Method for dehydrating a liquid food product in vacuum and while under vacuum exposing the completely dehydrated liquid to ultraviolet irradiation
U S 2 183 933	Dec 19 1939	J A Elderkin and E Hofman Ass to Chemical Products Co	Foods are antirachitically activated by the action of ozone
U S 2 202 611	May 28 1940	G C Supplee and J Dorcas Ass to The Borden Company and National Carbon Co	Method and apparatus for irradiation of milk in turbulent flow to increase the vitamin D content
U S 2 31 870	Feb 18 1941	W Baeckler Ass to Union Carbide & Carbon	Apparatus for open air irradiation of liquids such as milk by flowing the liquid in a substantially rectilinear direction along a smooth surface
U S 2 231 871	Feb 18 1941	W Baeckler Ass to Union Carbide & Carbon	Apparatus for open air irradiation of liquids such as milk by using a conical type of support for the liquid in order to maintain a constant rate of flow
U S 2 34 354	Mar 11 1941	H W Lilley and J Waddell Ass to DuPont	The process which comprises dissolving a provitamin D-containing material in an organic solvent adding a sugar amine exposing the solution to ultraviolet light and recovering a vitamin D concentrate

PATENT No	DATE	PATENTEE	ABSTRACT
<i>Conversion of Provitamins D to Vitamins D (Continued)</i>			
U S 2 243 632	May 27 1941	M L Johnson Ass to Vitamin Technologists	Production of vitamin D ₂ from ergo terol by irradiation with ultra violet light of 2536-3640 Angström Units
U S 2 260 823	Oct 28 1941	E S Bettis Ass to Pet Milk Co	Irradiation of milk in a thin film
B 265 910	April 7 1927	K. Hoefelmayer	Milk in concentrated form or in a curdled state is subjected to the action of artificially produced violet rays by which it is converted into an invalid food effective for the cure of certain diseases
B 266 101	Feb 24 1927	O A Elias	Method of manufacturing biscuits bread cakes and similar food products characterized by carrying out the baking process in the presence of artificially produced ultraviolet rays
B 270 296	April 28 1926	H C E Tillisch	Antirachitic vitamin by ultraviolet irradiation of oils or fats
B 285 083	Jan 24 1929	Merck	Manufacture of antirachitic preparations by esterifying the unsaponifiable constituents of yeast fat or of the corresponding extracts from ergot or similar lower fungi and exposing the esters to the action of an activating radiation or by first activating and afterwards esterifying the material
B 286 665	Sept 6 1928	Merck	Manufacture of antirachitic substances by irradiation of provitamins D in the presence of photochemical sensibilisers such as eosin or iodine
B 290 195	Mar 7 1929	C. Jaeger	Ultraviolet irradiation of dried bananas to produce an antirachitic food
B 293 255	Nov 28 1927	T D Kelly	Oils or emulsions are treated with beta rays and with ultraviolet rays to produce vitamins
B 295 757	Aug 23 1928	I S MacLean	Yeast is incubated in a solution containing phosphates and carbohydrates and the sterols or sterols and fats obtained are subjected to activating radiation
B 296 053	Aug 24 1927	A J Pacani Ass to M Richter	Process of treating materials to form antirachitic substances by various rays other than ultraviolet rays e g by x rays canal rays cathode rays etc
B 298 585	Dec 5 1928	Dry Milk Co	Ultraviolet irradiation of milk
B 302 980	Sept 24 1927	N Bendixen	Rotary device for treating liquids with rays or emanations
B 309 601	April 13 1928	E Oppenheim	Ultraviolet irradiation of chocolate
F 672 318	Mar 29 1929		

PATENT NO	DATE	PATENTEE	ABSTRACT
Conversion of Provitamins D to Vitamins D (Contd. used)			
B 313 553 G 5-4 874	June 14 1928 Sept 13 19 8	G Zecher Ass to N V Philip Gloel lampenfabrieken	Apparatus for irradiating substances with ultraviolet light
B 314 267	Aug 20 1928	I G	Apparatus for the irradiation of ergosterol in circulating solvent liquids
B 316 264	Sept 18 1929	O Red	Treatment of foods and liquids with short waves ultraviolet rays or x rays
B 318 068	Sept 2 1929	I M H Ibrson	Activation of provitamin D by metallic catalysts at elevated temperature
B 318 269	Sept 2 1929	I G	Production of antirachitic products by treating ergosterol or substances containing ergosterol with coronadischarges or with electric corpuscular rays or Roentgen rays while excluding oxygen
B 321 992 F 675 558	Nov 25 1929 May 10 1929	I G	Production of vitamin D by irradiation of sterols in the presence of substances which exert a protective action on the vitamin formed such as ether olefinic hydrocarbons or caustic alkalis
B 324 503	Mar 19 1930	Patent Treuhand Ges f elektr Glühlampen	Apparatus for ultraviolet irradiation of milk
B 325 470	July 12 19 9	J O Hickman and N V Hickman	In a process for the irradiation of flowing milk in thin layers the intensity of irradiation and rate of flow of the milk are regulated
B 342 500 F 697,367	Feb 15 1929 Jan 30 1930	T Reiter	Vitamin D by irradiation of ergosterol with light of wave lengths about 280 mμ
B 346 682	Oct 15 1929	V C From C D Rowley and A W Larkey	Simultaneous infra red and ultraviolet light irradiation of milk
B 357 2 3	Jan 4 1930	F F Tisdall	Wheat germ whole wheat and yeast treated with ultraviolet light are used in the manufacture of bread cakes etc
B 38,6 6 F 714 827	Dec 20 1930 April 4 1931	N V Philip Gloel fabrieken	Irradiation of ergosterol with ultraviolet light using a filter which absorbs light of wave lengths 312-313 mμ e g C ₂ H ₄
B 403 650	Jan 10 1934	J Waerham	Vitamin D containing soap by irradiation of soap containing ergosterol or of oils containing ergosterol followed by saponification
B 489 14	July 20 1938	H F Rost	Ultraviolet irradiation of thin films of provitamins D in solution or suspension in the presence of air with rays of wave length between 2600 and 3000 Angstrom Units
B 437,16	Dec 14 1938	Antirachitische Labora- torien	Antirachitic action of provitamin D by subcutaneous or topical non lumenous flow of electric

PATENT NO	DATE	PATENTEE	ABSTRACT
Conversion of Provitamins D to Vitamins D (Continued)			
B 498 068	Jan 3 1939	H F Glunz	Process for the irradiation of liquids and the sterile filling of containers therewith in which a zone of ultra violet rays is maintained within a vacuum chamber to irradiate the liquid as it is directed in a thin film past the zone the containers being filled with the treated liquid by direct connection with the interior of the vacuum chamber
G 502 728	April 3 1927	H Geffcken and H Richter	Foods such as milk are subjected to ultraviolet rays
G 523 257	Feb 8 1927	H Geffcken and H Richter Ass to A G f Elektrizitäts Ind	Apparatus for irradiating food stuffs
G 526 141	May 17 1929	H Geffcken and H Richter	Apparatus for irradiating foods with ultraviolet rays
G 530 877	Nov 4 1926	F Heinemann	Foodstuffs are vitaminized by ultraviolet radiation using a calc spar filter
G 545 080	Feb 25 1932	H Geffcken and H Richter	Irradiation of skimmed milk
G 556 716	Mar 7 1928	Merck	Stable aqueous colloidal solutions of irradiated ergosterol are obtained by irradiating ergosterol in its dispersed form and separating the unchanged ergosterol after irradiation
G 564 401	Nov 1 1927	F Kielwein	Apparatus for irradiating bread or yeast for incorporation into bread
G 608 277	Jan 19 1935		
G 564 736	Nov 22 1932	E Latacz	Apparatus for irradiation of liquids
G 566 744	Dec 20 1932		
G 567 333	Dec 31 1932	Hoffmann LaRoche	Production of water soluble sterol compounds by irradiating sterols in the form of their ester salts e.g. a salt of phthalic acid monoergosterol ester with ultraviolet light
G 568 900	Jan 5 1933	K. Hembold and Vitamin Fabrik	Irradiation of extracts of high viscosity
G 577 170	Aug 10 1933		
G 572 491	Mar 17 1933	A Schindler	Ultraviolet irradiation of milk
G 577 531	June 1 1933	C Kersten and O K. Schultz	Irradiation of skimmed milk followed by addition of the original cream
G 583 731	Sept 9 1933	I C	Production of an antirachitically active material by treating lumisterol or its esters with ultraviolet light and method of crystallizing the antirachitic product a the di nitro benzoate
G 622 373	Nov 27 1935	Leo-Werke	Vitamin containing cosmetics from wool fat by irradiation with ultraviolet light
G 624 325	Jan 17 1936	H Heitan and Kuntze's Verwaltungen G	Vitamins are produced in malt beer by light from a Mg arc
G 632 783	July 13 1936	Hanovia	Irradiation of milk with mercury vapor lamp
G 648 326	July 29 1937	E Irlitzky	Irradiation of milk in bottles

PATENT NO	DATE	PATENTEE	ABSTRACT
Conversion of Provitamins D to Vitamin D (Continued)			
G 673 852	Mar 30 1939	K Wolff and R Havemann	Method for obtaining transformation products of ergosterol and similar materials by irradiating with electrons from a glowing cathode in which the material to be activated is passed in thin layers over the anode
F 666 959	Oct 8 1929	Osa Part Ind Soc	Apparatus for the irradiation of liquids
F 667 660	Oct 19 1929	A Tribout	Pasteurization and irradiation of milk
F 677 010	Mar 3 1930	J Seip	Irradiation of beverages
F 677 111	June 17 1929	I G	Preparation of antirachitic substances by irradiation of provitamins at a temperature above 70° C
F 697 387	Jan 16 1931	T Renter	Conversion of ergosterol to vitamin D by means of light of wave lengths longer than 280 mμ
F 700 036	Nov 16 1929	H Labbé	Sterols extracted from cacao beans are activated by ultraviolet light
I 708 548	July 24 1931	N V Philips Gloeilampenfabrieken	Irradiation of provitamins in solution in special apparatus
I 722 61	Sept 20 1933	J Major	Vitamin-containing flour by special treatment of grain and ultraviolet irradiation
F 779 847	April 13 1935	C Devret & Co	Irradiation of cholesterol-containing foods
I 814 71	Jan 9 1940	Hermes Patentverwertungsgesellschaft	Method and apparatus for irradiating milk sprays in a carbon dioxide atmosphere
Austrian 118 762	Mar 15 1930	O Ried	The irradiation effect is enhanced by the addition of mineral substances e.g. ZnO
Austrian 118 764	Mar 15 1930	O Ried	Enhancement of the biological effect of irradiated substances by the addition of tryptophan
Austrian 137 455	May 11 1934	Leo Werke	Apparatus for irradiating wool fat or wool fat alcohols to enrich the vitamin D content
Belg 386 419	Mar 31 1937	Soc Réunis	Vitamin-containing foods by irradiation while stirring
Hung 100 696	Aug 4 1927	G Feher	Yeast is saponified and extracted. The isolated ergosterol is irradiated to form vitamin D
Hung 104 007	April 14 1931	A Jendrasik	Production of vitamin D from solid ergosterol in contact with a solution which dissolves the irradiated ergosterol and which solution is continuously removed
Vitamin D U S 1 503 134	Nov 24 1955	T F Zucker Ass to University Patents	Process of assaying the antirachitic strength of a substance by determining the pH value of the feces of experimental animals.

PATENT NO	DATE	PATENTEE	ABSTRACT
<i>Vitamins D (Continued)</i>			
U S 1 902 745	Mar 21 1933	A Windaus Ass to Winthrop	The antirachitic vitamin obtained by ultraviolet irradiation of ergosterol is purified by the formation of addition products of the non vitamin material with compounds of the maleic anhydride type
U S 1 902 785	Mar 21 1933	O Linsert	Process which comprises freeing an antirachitically active product from unchanged ergosterol by subjecting the product to the action of at least one mole of maleic or citraconic acid anhydride
R 370 743	April 14 1932	Ass to Winthrop	
R 405 321	Feb 5 1934		
G 565 900	Dec 7 1932		
G 578 021	May 6 1933		
U S 2 030 377	Feb 11 1938	O Linsert	Crystalline vitamin D ₂ from irradiated ergosterol by formation of the 3 5-dinitrobenzoate followed by saponification
G 603 038	Sept 22 1934	Ass to Winthrop	
U S 2 099 550	Nov 16 1937	A Windaus and F Schenck	Process for the purification of vitamin D ₂ obtained by activation of 7 dehydro-cholesterol by esterification with meta dinitro benzoyl chloride fractional crystallization of the esters formed and saponification of the meta dinitro benzoate of the vitamin D ₂ . Purification may also be accomplished by precipitation of 7 dehydro-cholesterol as the digitonide or by the formation of addition products with compounds of the maleic anhydride type
R 491 653	Sept 6 1938	Ass to Winthrop	
G 661 686	June 24 1938		
U S 2 179 560	Nov 14 1939	S E Miller Ass to General Mills	Process of concentrating vitamin D from either naturally occurring or synthetic vitamin D concentrates by dissolving vitamin D containing material in an organic solvent passing the solution through tricalcium phosphate whereby a major portion of the vitamin D is adsorbed by the phosphate washing the phosphate with an organic solvent and then separating the vitamin D from the solvent by distillation
U S 2 216 719	Oct 8 1940	A G Boer J van Niekert A van Wijk and E H Reenick Ass to Hartford National Bank & Trust Co	Process for producing a new chicken active vitamin D by activation of a new provitamin D derived from periwinkles
R 315 277	Sept 25 1930	Société des Usines Chimiques	Separation of ergosterol from its crude irradiation products by crystallization from an organic solvent particularly alcohol acetone and ethyl acetate
I 698 040	June 11 1930	Rhone Poulenc	
D 464 066	April 12 1937	N V Philips Gloeilampen	Process of producing a preparation which has a high antirachitic activity for chickens wherein the unsaponifiable fraction or the sterol fraction of duck eggs is irradiated with ultraviolet light. The provitamin D is identical with 7 dehydro-cholesterol
G 678 533	July 17 1939	fabrieken	
I 815 545	July 13 1937		

PATENT NO	DATE	PATENTEE	ABSTRACT
Vitamins D (Continued)			
B 482 880	June 17 1937	Eastman Kodak	Isolation of a new vitamin D by molecular distillation of degassed vitamin D containing oils
B 491 007	Aug 23 1938	Eastman Kodak	Esterification of vitamin D or of provitamin D with unsaturated higher fatty acids especially with linolenic acid. The provitamin D esters are activated to vitamin D ester
B 517 214	Jan 24 1940	Eastman Kodak	High vacuum short path distillation of natural vitamin D esters resulting in the separation of 5 or 6 different antirachitic materials
G 550 496	Apr 127 1930	L. Brauer and H. Seel	Vitamins by oxidation of cholesterol with benzoyl peroxide
G 659 882	May 17 1938	H. Brockmann Ass to I. G.	Isolation of the antirachitic vitamin from natural products
G 672 000	Feb 18 1939	H. Fincke Ass to Gebrüder Stollwerck	Fats with high vitamin D content are obtained by extracting ground cocoa shells with cocoa butter
Can 379 424	Feb 9 1939	L. Yoder Ass to Iowa State College Alumni Assoc.	Antirachitic substance by heating cholesterol with a mixture of concentrated sulfuric acid, acetic anhydride and acetic acid
Derivatives and Utilization			
U S 1 874 653 B 334 002	Sept 22 1931 July 1 1929	E. Brauchl Ass to Hoffmann-La Roche	Irradiated ergosterol is stabilized by the addition of a small quantity of a dihydroxy phenol such as hydroquinone
U S 1 974 808	Sept 25 1934	C. F. Dietz Ass to Commander Larabee Corp.	The oil from a germ bearing cereal grain is irradiated to form vitamin D and is incorporated into flour
U S 2 010 792	Aug 6 1935	J. Segel Ass to A. M. Sloan	Colloidal dispersion of sterols in water containing bone meal and alfalfa extract
U S 2 072 464	Nov 26 1935	L. A. Hall Ass to C. L. Griffith	An emulsified vitamin D concentrate containing gum tragacanth and gum acacia, the emulsion having a pH of 5.5 to 6.0
U S 2 030 792 B 436 713 B 469 150 G 647 261	Feb 11 1936 Oct 16 1935 July 20 1937 Feb 27 1937	C. W. Hooper Ass to Winthrop	Propanediols and butanediols as solvents for fat soluble vitamins especially for vitamins D
U S 2 070 117 G 674,231	Feb 9 1937 Jan 15 1936	O. Dalmer and F. v. Werder Ass to Winthrop	Hydrogenation of tachysterol-dinitrobenzoate to form dihydro-tachysterol
U S 2 150 316	Mar 14 1939	A. E. Briod and B. R. East Ass to National Oil Products	Vitamin D milk is prepared by homogenizing a cod liver oil concentrate with cream or evaporated milk followed by canning and sterilization
U S 2 175 340	Oct 10 1939	J. W. D. Chesney Ass to New Discoveries Inc.	A vitamin D concentrate is added to a product such as beer containing at least 0.25% of alcohol and dissolved CO ₂

PATENT NO	DATE	PATENTEE	ABSTRACT
Derivatives and Utilization (Continued)			
U S 2 180 969	Jan 2 1940	H E Schultze Ass to Winthrop	Preparation of clear aqueous therapeutic solution of vitamin D with triolein polyethylene glycol oleyl ether and physiological NaCl solution
U S 2 194 188	Mar 19 1940	G C Supplee Ass to The Borden Co	Method for producing a vitamin D protein symplex of enhanced antirachitic activity
U S 2 228 491	Jan 14 1941	F v Werder Ass to Winthrop	Isolation of pure dihydro-lachysterol
U S 2 245 418	June 10 1941	R C Sherwood and C G Ferrari Ass to General Mills	Production of a sterile emulsion of evaporated milk fortified with a concentrate of vitamin D ₂ by dissolving activated ergosterol in butter fat and then dispersing the butter fat concentrate in evaporated milk and canning and sterilizing the resulting product
U S 2 264 320	Dec 2 1941	O Linsert Ass to Alba	Vitamin D ₂ double compounds with cholesterol cholestanol or coprosterol
U S 2 265 320	Dec 9 1941	R C Sherwood and C G Ferrari Ass to General Mills	Emulsion of vitamin D ₂ in evaporated milk
B 400 791	Feb 15 1934	Bell and Sons Ltd and J Sowler	Vitamin D containing oils are absorbed in flour or other carriers of porous nature and mixed with mineral matter for animal feeds
B 406 629 F 737 234	Not accepted May 17 1932	N V Philips Gloeilampenfabrieken	Vitamin D is preserved by adding oil or fat to a solution of ergosterol before during or immediately after irradiation in the absence of oxygen
B 412 530	July 11 1934	N V Philips Gloeilampenfabrieken	Poultry feeds containing vitamin D ₂
B 440 888	April 13 1935	Standard Brands	Increase in vitamin D content of eggs by feeding hens vitamin D
G 495 400	June 30 1927	Hoffmann LaRoche	Sterols other than cholesterol and ergosterol are water solubilized by the formation of mono esters of dicarboxylic acids
G 501 904 Add to 490 450	Aug 6 1927	Hoffmann LaRoche	Photo activated ergosterol is water solubilized by the formation of mono-esters of dicarboxylic acids

VITAMINS E

Isolation			
U S 2 188 878	Jan 30 1940	C L Lautenschläger and F Lindner	Process for preparing crystallized vitamin E allophanates by esterification with cyanuric acid followed by purification by adsorption on aluminum oxide followed by elution
G 601 474	Oct 15 1937	Ass to Winthrop	

PATENT NO	DATE	PATENTEE	ABSTRACT
Isolation (Continued)			
U S 2 903 400 B 531 2 4	June 4 1940 Dec 31 1940	J E Andrews Ass to General Mills	Vitamin L concentrate from wheat germ oil by catalytically hydrogenating the oil extracting the hydrogenated oil with alcohol and further concentrating by separating the sterols and glycerides at low temperatures saponifying and extracting
U S 2,263 550 B 31 920	Nov 18 1941 Dec 31 1940	J S Andrews Ass to General Mills General Mills	Preparation of vitamin E concentrate from wheat germ oils by esterification with a monohydric alcohol followed by distillation of the vitamin E ester Process for preparing a concentrate of vitamin E from vegetable oils by ester interchange of the glycerol esters with a lower aliphatic monohydric alcohol to yield glycerol and esters of monohydric alcohols followed by separating the glycerol and the sterols and saponifying the remaining mixture
Synthesis			
U S 2 208 585 B 528 371	July 23 1940 Sept 17 1941 Oct 28 1940	P Karrer Ass to Hoffmann LaRoche P Karrer Ass to Hoffmann LaRoche	Synthesis of vitamin E by condensation of alkylated hydroquinones with halogen derivatives of phytol Separation of alpha tocopherol from its racemic synthetic mixture comprising the treatment with 3 bromocamphor sulphonic acid chloride isolation of the condensation product and hydrolysis to yield the free pure alpha tocopherol
U S 2 30 659 B 529 082	Feb 4 1941 Nov 13 1940	F v Werder Ass to Merck L I Smith and H E Ungnade Ass to University of Minnesota	Process for the production of alpha tocopherol comprising condensing trimethyl hydroquinone with phytol in the presence of phosphorus pentoxide Manufacture of tocopherol like compounds by reacting hydroquinones or their monoethers with dienes in the presence of an acid substance
U S 2 245 147 B 529 081	June 10 1941 Sept 27 1940	W John and F Günther Ass to Merck Hoffmann LaRoche	Synthesis of compounds of the general formula of vitamin E by reacting 3,4-dihydro coumarin with a mixture of methyl magnesium halogenide and the Grignard compound from a higher halogenated hydrocarbon Process for the manufacture of di tocopherol in which methyl substituted hydroquinones are reacted with phytol isophytol phytyl aldehyde or 3 halogeno-dihydro-phytyl bides in the presence of zinc chloride or formic acid
U S 2 249 054 B 529 082 (Add to B 529 081) B 5884	July 13 1941 Nov 13 1940 Sept 27 1940	L I Smith and H E Ungnade Ass to University of Minnesota Hoffmann LaRoche	Condensation of trimethyl hydroquinones and alkyl halides (with the exception of phytyl halides)
B 7,330	Oct 8 1940	Hoffmann LaRoche	

PATENT NO	DATE	PATENTER	ABSTRACT
Synthesis (Continued)			
B 578 372	Oct 28 1940	Hoffmann LaRoche	Condensation of alkyl hydroquinones with halides of alpha beta unsaturated carboxylic acids in the presence of an acid condensing agent
B 529 081	Nov 13 1940	University of Minnesota	Manufacture of coumarins and chromanes by condensation of hydroquinones alkylated or not alkylated with substituted or unsubstituted alkyl halides
F 855 414	May 10 1940		
B 532 364	Jan 22 1941	Hoffmann LaRoche	Acetyl <i>dl</i> alpha tocopherol by acetylation of <i>dl</i> alpha tocopherol
B 377 774	July 7 1941	Hoffmann LaRoche	Manufacture of tocots from phytol halides and alkylated hydroquinones
B 39 697	Sept 27 1941	Hoffmann LaRoche	Synthesis of vitamin E from trimethyl hydroquinone via the monoalkylene ether (e g of phytol) by intramolecular condensation
B 540 907	Nov 5 1941	Hoffmann LaRoche	Synthesis of ethers and esters of tocots by condensation of phytol compounds with mono ethers or mono-esters of dimethyl hydroquinones
B 541 008	Nov 10 1941	Hoffmann LaRoche	Manufacture of ring homologues of vitamin E by condensation of phytol compounds with alkylated hydroquinones
B 541 011	Nov 10 1941	Hoffmann LaRoche and J F Pollak	Synthesis of vitamin E by rearrangement of trimethyl hydroquinone mono-phytyl ether followed by condensation under acidic conditions
G 374 142	April 20 1923	L Claisen	Synthesis of chromanes by condensation of phenols with butadiene hydrocarbons in the presence of acidic condensing agents
G 703 957	Mar 20 1941	O Hromatka Ass to Merck	Synthesis of vitamin E by condensation of 3 amino-6 oxy 1,4 trimethyl benzene with phytol compounds followed by conversion of the amino into a hydroxyl group
Intermediates for the Synthesis			
U S 2 229 573	Jan 21 1941	F Jung	Trimethyl hydroquinone from trimethyl quinone by catalytic hydrogenation
C 683 908	Nov 18 1939	Ass to Merck	
Add to C 676 198			
U S 2 229 574	Jan 21 1941	F Jung Ass to Merck	Xylohydroquinone by catalytic hydrogenation of xyloquinone
U S 2 249 936	Oct 21 1941	F Jung	Duro-hydroquinone from duroquinone by catalytic hydrogenation
G 676 198	May 30 1939	Ass to Merck	
B 308 292	June 28 1939	Hoffmann LaRoche	Manufacture of phytol bromide
B 537 793	July 7 1941	Hoffmann LaRoche	Condensation of trimethyl hydroquinone with acetyl phytol to yield phytol trimethyl or mono acetyl hydroquinone

PATENT NO	DATE	PATENTER	ABSTRACT
Derivatives and Utilization			
U S 2 167 002	July 25 1939	A. J. Pacini Ass to U S Vitamin Corp	Composition of matter claims for vitamin E and magnesium distributed therein in an amount not less than 0.00%
U S 2,212 531	Aug 27 1940	F v Werder Ass to Merck	Process claims for the reaction of durohydroquinone with long chain alkyl halides alkylene halides and hydro aromatic halides to form compounds of the type $C_n(CH_2)(OH)OR$ R = 6-14 carbon atoms Product claims for the latter compounds with vitamin E activity
U S 2 219 532	Aug 27 1940	F v Werder Ass to Merck	Process claims for the reaction of trimethyl hydroquinone with long chain alkyl halides alkylene halides and hydro aromatic halides to form compounds of the type $C_nH(CH_2)(OH)OR$ R = 6-14 carbon atoms Product claims for the latter compounds with vitamin E activity
U S 2 216 841 B 506 609 B 527 006	Oct 8 1940 May 31 1939 Sept 30 1940	O Isler Ass to Hoffmann LaRoche	Composition of matter claims to duro hydroquinone mono phenyl ether and process for preparation by reacting durohydroquinone with a phenyl halide in the presence of an alkali metal carbonate
U S 2 231 125 B 536 609	Feb 11 1941 May 21 1941	P Karrer Ass to Hoffmann LaRoche	Composition of matter claim for tocopherol-oleate and stearate
U S 2 358 841	Mar 25 1941	W John and O Dalmer Ass to Merck	Process for the manufacture of monoethers of trimethyl hydroquinone by treatment with aliphatic or aromatic saturated or unsaturated alcohols ester or halides Product claims for the octadecyl and no decyl mono-ethers.
U S 2 245 480 B 579 023 G 702 491	June 10 1941 Nov 12 1940 Feb 8 1941	P Karrer Ass to Hoffmann LaRoche	Process for the manufacture of condensation products from beta tocopherol and alpha beta unsaturated alkyl halides
U S 2 247 364 G 704 171 B 517 932	July 1 1941 Mar 25 1941 Feb 13 1940	E Fernholz Ass to Merck Ciba	Mono and dialkyl ethers of durohydroquinone Manufacture of mono ethers from alkylated para dihydroxy benzenes and aliphatic alcohols containing 10 or 11 carbon atoms forming a branched carbon chain to which may also be linked an aliphatic residue Also esterification of the free hydroxyl group of the reaction product

VITAMIN H

U S 2 193 533	Mar 12 1940	F Schults Ass to Wethrop	Vitamin H is liberated from tissue protein by hydrolysis at elevated temperatures and extraction with organic solvents
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PATENT NO	DATE	PATENTEE	ABSTRACT
U S 2 02307	May 28 1940	L E Hooper	Concentration of vitamin H by adsorption of an alcoholic solution on activated carbon followed by elution with alcohol benzene alcohol or acetone
B 409 524	Jan 11 1937	I G	Purification of vitamin H preparations by electrodialysis
G 645 414	May 27 1937		
B 463 698	April 5 1937	F Schultz	Extraction of vitamin H from animal organs by energetic hydrolysis followed by acetone addition which precipitates impurities and leaves the vitamin in solution
G 664 088	Aug 19 1938	Ass to I G	
G 661 435	Oct 15 1937		
B 481 981	May 9 1938	I G	Treatment of seeds with biotin or derivatives in various carriers
F 824 878	Feb 17 1938		
G 661 929	June 30 1938	I G	Biotin extracts from yeast seeds germs egg yolk etc by precipitation of impurities in crude extracts by means of Reincke salt picronic or oxalic acid or by acetylation Precipitation of biotin with phosphotungstic acid or mercuric chloride
G 670 098	Jan 11 1939	I G	Precipitation of biotin with H_2PtCl_6
G 670 922	Jan 27 1939	I G	Isolation of biotin after previous esterification
Continuation of 670 098			

VITAMINS K

U S 2 233 779	Feb 25 1941	S Ansbacher E Fernholz and M L Moore Ass to Squibb	Process for concentrating vitamin K by adsorption on activated charcoal followed by elution
B 333 513	Feb 14 1941	St Louis University	Isolation of vitamins K by adsorption on a base exchange silicate e g sodium aluminum silicate followed by extraction with non polar solvents e g benzene
B 339 471	Sept 11 1941	Parke Davis	Manufacture of 2 methyl 4 amino naphthol from 1 4 naphthoquinone or nitro-methyl naphthalene
B 341 138	Nov 13 1941	Hoffmann LaRoche	Manufacture of acid succinic acid esters of alkylated 1 4 naphtho hydroquinones

VITAMIN P

U S 2 224 807	Dec 10 1940	M Brockmühl and E Bartholomäus Ass to Winthrop	Process for the production of glucosides of polyhydroxy flavanones
B 486 898	June 13 1938	A Szent Györgyi	Process for the extraction of flavones from vegetable material by means of an organic solvent miscible with water precipitation with an alkali or earth alkali metal hydroxide followed by acid decomposition
Can 398 341	July 29 1941	Ass to Winthrop	

CHOLINE

PATENT NO	DATE	PATENTEE	ABSTRACT
U S 1904 (X) B 315 713	April 18 1933 Jan 13 1930	G Roy Ass to Rhône Poulenc	Solution of acetylcholine salts in ethylene glycol monoacetate or glycol mono- or diacetate
U S 197443	May 8 1934	J K Clie Ass to Merck	Salt of acetylated choline
U S 1957461	May 8 1934	L W Jones and R T Major Ass to Merck	Acetylcholine salts
U S 2043463	Aug 4 1936	R T Major and J Kline Ass to Merck	Salts of beta alkylated choline alkyl ethers
B 8031/14	Mar 30 1914	J Y Johnson	Salt of choline
B 379 60 F 736 107	Aug 25 1932 April 11 1932	F Körner	Preparation of salts of choline
C 290 3	Aug 12 1913	Hoffmann La Roche	Non hygroscopic salts of choline with halohydroxybenzoic acids
G 40 72	May 1 1918	Chem Werke Crenzach	Arsonium compounds of the choline type
G 590 311	Jan 10 1934	Merck	Quaternary salts of choline derivatives
C 33258	Feb 23 1934	L Glucksmann	Choline salts of bile acids
C 638 641	Nov 20 1936	Hoffmann La Roche	Alcoholic solutions of hygroscopic salts of acetylcholine

RECOMMENDED DIETARY ALLOWANCES^a

Recommended by the Food and Nutrition Board National Research Council

Group	Calo ries	Pro tein g	Cal cium g	Iron mg	Vita min A ^c I U	Thia min (B ₁) mg ^b	Ribo fla vin mg	Niacin (nico tinic acid) mg	Ascor bic acid mg ^b	Vita min D I U
Man (70 kg) Seden tary	2500					1 5	2 2	15		
Modera tely active	3000	70	0 8	12	5000	1 8	2 7	18	75	
Very active	4500					2 3	3 3	23		
Woman (56 kg) Seden tary	2100					1 2	1 8	12		
Modera tely active	2500	60	0 8	12	5000	1 5	2 2	15	70	
Very active	3000					1 8	2 7	18		
Pregnancy (latter half)	2500	85	1 5	15	6000	1 8	2 5	18	100	400-800
Lactation	3000	100	2 0	15	8000	2 3	3 0	23	150	400-800
Children up to 12 years										
Under 1 yr ^d	100/kg	3 4/kg	1 0	6	1500	0 4	0 6	4	30	400-800
1-3 yrs ^e	1200	40	1 0	7	2000	0 6	0 9	6	35	/
4-6 yrs	1600	50	1 0	8	2500	0 8	1 2	8	50	
7-9 yrs	2000	60	1 0	10	3500	1 0	1 5	10	60	
10-12 yrs	2500	70	1 2	12	4500	1 2	1 8	12	75	
Children over 12 years										
Girls 13 15 yrs	2500	80	1 3	15	5000	1 4	2 0	14	80	
16-20 yrs	2400	75	1 0	15	5000	1 2	1 8	12	80	
Boys 13- 15 yrs	3200	85	1 4	15	5000	1 6	2 4	16	90	/
16-20 yrs	3800	100	1 4	15	6000	2 0	3 0	20	100	

Footnotes to Table

^a Tentative goal toward which to aim in planning practical dietaries can be met by a good diet of natural foods. Such a diet will also provide other minerals and vitamins the requirements for which are less well known.

^b 1 mg thiamin equals 333 I U. 1 mg ascorbic acid equals 20 I U.

^c Requirements may be less if provided as vitamin A, greater if provided chiefly as the provitamin carotene.

^d Needs of infants increase from month to month. The amounts given are for approximately 6-8 months. The amounts of protein and calcium needed are less if derived from human milk.

^e Allowances are based on needs for the middle year in each group (as 2, 5, 8, etc.) and for moderate activity.

^f Vitamin D is undoubtedly necessary for older children and adults. When not available from sunshine, it should be provided probably up to the minimum amounts recommended for infants.

Further Recommendations Adopted 1942

The requirement for iodine is small, probably about 0.002 to 0.004 milligram a day for each kilogram of body weight. This amounts to about 0.15 to 0.30 milligram daily for the adult. This need is easily met by the regular use of iodized salt; its use is especially important in adolescence and pregnancy.

The requirement for copper for adults is in the neighborhood of 1.0 to 2.0 milligrams a day. Infants and children require approximately 0.05 per kilogram of body weight. The requirement for copper is approximately one tenth of that for iron.

The requirement for vitamin K is usually satisfied by any good diet. Special consideration needs to be given to newborn infants. Physicians commonly give vitamin K either to the mother before delivery or to the infant immediately after birth.

AUTHOR INDEX*

A

- Abbasy M A 33^v
 Abbasy M A Harrs L J and
 Filman P 334
 Abbasy M A Hill N G and
 Harrs I J 334
 Abbott O D and DeMasters
 C U 515 548
 Abderhalden E and Fodor A
 260
 Abderhalden E and Wert
 heimer W 142
 Abraham E P 170
 Abraham I P and Adler E
 174
 Abt A P and Farmer C J
 334 337
 Abt A F Farmer C J and
 Epstein I M 3 6
 Abt A F see Farmer C J
 335
 Ackermann D 226 244 263
 Ackerson C W see Mussehl
 F E 344
 Acton H W Ghosh S and
 Dutt A 130
 Adams A B see Iscovesco H
 564
 Adastone F R 458 461 463
 Addicott F T and Bonner J
 243
 Addinall C R 128 13^v
 Adkins H see Floyd L W
 304
 Adler E Das H H and
 Euler E v 228
 Adler E and Euler H v
 173 174 189
 Adler E Euler H v and Gün
 ther G 174
 Adler E Euler H v Günther
 G and Plass E D 174 2⁷⁸
 Adler E Günther G and
 Everett J E 2⁷⁸
 Adler E Hellström H and
 Euler H v 2⁷³
 Adler E and Michalis M
 174
 Adler E and Sreenivasaya M
 228
 Adler F see Abraham I P
 174
 Adler L see Euler H v
 154 155 174 18² 183 18³
 189 2⁷⁸ 230 233 23^v 237
 Aeschlimann J A see Mills
 W H 114
 Aggeler P M Lucia S F and
 Goldman L 506 507
 Agid R see Miz B 142
 Agopian L A 290 555 587
 Ahlström L see Euler H v
 88
 Ahmad B 54 86 292 301 329
 Ahmad B and Malik K 55
 Ahrens P B and Gorkow R
 2⁷⁴
 Ahrens G Fernholz E and
 Stoll W 375
 Albeaux Fernet M see Quer
 ido A 249
 Albers H Schlenk F and
 Euler H v 232
 Albers H s e Euler H v 220
 230 234 585
 Albert H and Schlenk F
 586
 Aidersley J B and Burkhardt
 G N 402
 Alderson W L see Milas
 N A 401
 Alexander F R 555 591
 Allchorne E see Bacharach
 A L 437 454
 Allen A W 373
 Allen F H see May C D 5^r
 93 94
 Allen R see Bourne C 3⁷⁴
 370
 Allen T J 553
 Allison F E Hoover S R and
 Burk D 469 474 475
 Allison F E and Minor F W
 474
 Allyn L B 555
 Almquist H J 48² 485 494
 495 499 509
 Almquist H J and Klos
 A A 48² 485 492 494 497
 499 501 50²
 Almquist H J Mecchi E
 Stokstad E L R and
 Manning P D V 542
 Almquist H J Pentler C F
 and Mecchi F 483 491
 Almquist H J and Stokstad
 E L R 483 484 50²
 Almquist H J Stokstad E L
 R Mecchi E and Manning
 P D V 542
 Almquist H J see Klose A A
 483
 Almquist H J see Lepkovsky
 S 188 191
 Almquist H J see Stokstad
 E L R 543
 Althausen T L see Stockholm
 M 134
 Aman H 559
 Amberg E J see Holmes
 H N 330 334
 Ambros O see Walz E 594
 Ammon R and Dirscherl W
 14
 Andersag H and Westphal K
 100 108 11² 116 118 127
 576 577 579
 Andersag H see Kuhn R 202
 203
 Andersag H see Westphal K
 577 579 584
 Andersen A 463
 Andersen A and Nightingale
 E 78
 Andersen A A see Wood H
 G 193
 Anderson B A see Clot M
 M 4⁷⁷
 Anderson H D Elvehjem
 C A and Gonce J E 462
 Anderson J A see Stresser
 A V 544
 Anderson R J Lothrop W
 C and Creighton M M
 276
 Anderson R J and Newman
 M S 490
 Anderson R J and Roberts
 E G 276
 Anderson W T and Macht
 D I 40³
 Andrews J S 607
 Andrus W D and Lord J W
 506
 Andrus W D Lord J W and
 Moore R A 506
 Anger V see Christiani A von
 367 368

- Angus T C Askew T A
 Bourdillon R B Bruce
 H M Callow R K Fischer
 mann C Philpot J S L
 and Webster T A 370
 Ansbacher S 283 284 286
 502-504 507 508
 Ansbacher S and Fernholz
 E 482 496 499
 Ansbacher S Fernholz F and
 Dolliver M A 494 491
 Ansbacher S Fernholz F and
 Moore M L 610
 Ansbacher S Fernholz I and
 MacPhallamy H B 489
 Ansbacher S Flanagan G E
 and Supplee G C 580
 Ansbacher S and Landy M
 286 474
 Ansbacher S Supplee G C
 and Bender R C 106
 Ansbacher S see Fernholz E
 499 496 503
 Ansbacher S see Martin G J
 280 283 286
 Ansbacher S see Supplee
 G C 182 409
 Ansbacher S see Wisansky
 W A 183 283 286
 Antener I 298
 Antopol W and Schotland
 C E 214
 Antopol W and Unna K 214
 Antopol W see Glick D 143
 Antopol W see Scudi J V
 210 219
 Antopol W see Unna K 210
 Appel H 308
 Aquirezabla J see Randon
 L A 183
 Archer H E and Graham G
 335
 Arcus C L and Silva S S
 292 293 298
 Arloing F Morel A and Jos-
 serand A 593
 Armentanó L 517
 Armentanó L Bentsath A
 Béres T and Rusznák I
 513 518
 Armentano L Bentsath A
 Béres T Rusznák I and
 Szécs Györgyi A 513
 Armentanó L Ratz E B and
 Rusznák I 517
 Arnim S see Eliot M M 47
 Arnold A and Elvehjem C A
 131 541
 Arnold A Kline C L Elve-
 hjem C A and Hart E B
 541
 Arnold A see Elvehjem C A
 101
 Arnold A see Weinstock H
 H 463
 Arnold G D 555
 Arnold R T and Larson R
 491
 Arnon D I 133
 Aron W 333
 Ascham J K see Schneider
 H A 198 211
 Aschehoug V see Bakke A
 253
 Ashburn L L 271
 Ashe W F see Spies T D
 192 214 216 249
 Ashford C A 430
 Ashford C A see Wilkinson
 J F 334
 Askew F A Bourdillon R B
 Bruce H M Callow R K
 Philpot J S I and Webster
 T A 380
 Askew F A Bourdillon R B
 and Webster T A 369
 Askew F A see Angus T C
 370
 Astra A see Norbin A B O
 558
 Atkin L see Schultz A S 131
 134 148 211 212
 Atwood A G 530
 Auchinachie D W and Fraser
 A H H 432
 Auerbach M E 129
 Auhagen E 123 565 583
 Auhagen E see Windaus A
 377
 Ault R G Baird D K Car-
 rington H C Haworth W
 N Herbert R W Hirst E
 L Percival E G V
 Smith F and Stacey M
 291 308
 Austin F L see Evans H M
 449 451
 Austin W C and Humoller
 F L 170
 Auwers K v and Wolter I
 356
 Axelrod A E and Llivchjem
 C A 188 234 249
 Axelrod A E Madden R J
 and Elvehjem C A 244
 Axelrod A E Sober H A and
 Elvehjem C A 199
 Axelrod A L Spies T D and
 Elvehjem C A 188 199
 244
 Axelrod A C see Wagner
 J R 183
 Axtmayer J H see Sherman
 H C 99 106
 Aykroyd W R and Roscoe
 M H 920
 Aylward F A Channon H J
 and Wilkinson H 7 546
 1
 B
 Babcock S H and Jukes
 T H 200
 Babcock S H see Dalt F S
 69 271
 Babcock S H see Spies T D
 468
 Bacharach A L 283 460
 Bacharach A L and All-
 chorne E 454
 Bacharach A L Allchorne
 E and Glynn H E 437 454
 Bacharach A L and Smith
 E I 99
 Bachar F A see Trenner
 H R 500
 Bachstet M and Cavallini
 G 30
 Bacon W E see Tourtellotte
 D 417
 Badmond C A 328
 Baedler W 599
 Bahler G P see Lampen J O
 470
 Bailly O and Netter R 38
 Baird F D and Greene D J
 410
 Baird D K Haworth W N
 Herbert R W Hirst E L
 Smith F and Stacey M
 291 308
 Baird D K see Ault R G
 291 308
 Baker A Z and Wright M D
 416
 Baker L E G
 Bakke A Aschehoug V and
 Zbinden C 203
 Ball E G 175 293
 Bancroft F W see Quirk
 A J 507 508
 Bandier E 240 244 247 248
 Band er E and Hald J 240
 Banerjee S 336
 Banerji G G and Harris L J
 148
 Banga I and Szécs Györgyi
 A 104 177
 Banga I Szécs Györgyi A
 and Vargha L 104
 Barac G 391
 Barbet E A 561
 Barborka C J see Friedemann
 T E 248
 Barbour A D 570
 Barber G Bergel F and Todd
 A R 111 120 10 178
 Barnes D J and Carpenter
 A D 499
 Barnes D J see Eliot M M
 412
 Barnett H M 569
 Barnett H M Frohning W
 O and Germann A F 569
 Barnett M see Zucker T F
 343
 Barnum G L 463
 Barnum G L see Roe J H
 397
 Baron E see Brunner O 150
 Barr T Hebron I M Parry
 E G and Sprung T S
 360

- B rrie M M O 456 459 46
 B rron C G and Hastings
 A B 160
 Barron E G and Lyman
 C M 138 140 144
 B rron E S G Lyman C M
 Lipton M A and Goldinger
 J 139 141
 Bartenstein L 290
 Barthen C L 555
 Bartholomäus E s e Brock
 mühl M 610
 B ritley M A see Robbins
 W J 16 133
 Barton R W and Cox W M
 556
 Bartow E and Walker W W
 587
 Bartow E Walker W W and
 Hogl n F A 277
 Basu K P and Nath M C
 317
 Bateman W G 405
 Batty J W Burawoy A
 Harper S H Helbron I M
 and Jones W E 70
 Batty J W see Helbron I
 M 52
 B uer E s e Euler H v 77
 Bauernfeind J C and Norris
 L C 09 270
 Bauerfeind J C Schumacher
 A E Hodson A Z Norris
 L C and H user G F 3
 B um H M see Pavcek P L
 275 280 477
 Baum W S te Cl use S W
 89
 B umann C A and Foster
 E C 89
 Baumann C A and Moore T
 91
 Baum nu C A Ri ng B M
 and Ste nbock H 55
 Baumann C A Steenbock H
 De son W M and Rup l
 I W 80
 Baumann C A ee J cob
 H P 547
 Baumann E J 793
 Baumann F J and Metzger
 N 91
 Baumberger J P 506
 Baumgarten P and Dornow
 A 17
 Baxter J C 569
 Baxter J G Gray E L and
 Tinker O A 61
 Baxter J C H rris P L
 H ckman K C D and Robe-
 son C D 60
 Baxter J G and Robeson C
 D 60 61 75 81
 B y F see Vogt Möller F 463
 Beach E F and White A
 549
 Bean W B see Spes T D
 191 214 216 245 246
 Beard H H Burk R F
 Thompson H E and Gold
 blatt H 369
 Beardsl F C 596
 Bech P F 415
 Bechdel S I Honeywell H E
 Dutcher R A and Knutsen
 M H 101 150
 Bechtel H E and Hoppert
 C A 403 406
 Becker B see Euler H v 167
 181 239
 Becker B see Karrer P 163
 167 191
 Becker G see Widenbauer F
 128
 Bedford F see Erdmann E
 534
 Beeston A W Channon H J
 Loach J V and Wilkinson
 H 543
 Behr L D see Clarke H T
 962
 Behring H v see Schönheimer
 R 366
 B ll M E see Drummond
 J C 85
 Beler W B Hauck H M
 and Storv ck C A 333
 Bendas H ee Karrer P 179
 Bender R C a d Supplee
 G C 211
 Bender R C see Ansbacher
 S 156
 Bender R C s e Supplee
 G C 183 409 556
 Bend en N 600
 Benford F s e Knudson A
 369
 Benjamin H R 43 474
 Benjamin H R and Hess
 A F 43 474
 Benjamin H R see Hess A F
 424
 Benko A see Rusznayk I 518
 Bennett W G 594 596
 Bentsäth A and Das N B
 16
 Bent äth A Rusznayk I and
 Sent György A 513 516
 Bentsäth A see Armentan
 I 113 518
 Benz I see Ful r H v 167
 181
 Benz F ee Karrer P 163
 165 167 181 38
 Ber nd H and Fischer M
 335
 Bérés T see Arm ntanó L
 513 18
 Berg M A 258
 Bergel F Copping A M
 Jacob A Todd A R and
 Work T S 445
 Bergel F Todd A R and
 Work T S 441 443
 Bergel F see B rger G 111
 120 16-1 3
 Bergel F see Todd A R 111
 437 438 448
 Bergen v 8
 Berger E see Brederick H
 170
 Berger R see Pag t M 322
 Bergh A A H van den Muller
 P and Broekmeyer J 38
 Bergström S see Winter
 ste ner O 360
 Bernardin L 559
 Bernhard A see Pickhardt
 O C 330
 Bernhard K 28
 Bernhard P see Woker G 36
 Bernheim F and Bernheim
 M L C 506
 Bernheim F and Felsovanyi
 A v 45
 Bernhim M L C see Bern
 heim F 506
 Bernstein R b 36
 Bc s n T 173 548
 Be trand G 304
 Be ey O A 185 318 319
 Bessey O A and Wolbach
 S B 83 183 191
 Bessey O A see H t ngs
 A B 190
 Bessey O A see Pinkerton
 H 190
 Bessey O A see Waugh W A
 293
 Bessey O A see Wolbach S B
 9
 Best C H Grant R and
 R dout J H 543
 Best C H Hershey J M and
 Huntsman M E 543 544
 Best C H and Huntsman
 M E 543 544
 Best C H Huntsman M E
 Melheny E W and Ridout
 J H 543
 Best C H and Ridout J H
 544 546
 Best C H see MacLe n D L
 545
 Bethke R M Kennard D C
 and Sassaman H L 418
 Bethke R M Krauss W E
 Record P R and Wilder
 O H M 418
 Bethke R M Record P R
 Wilder O H M and Kirk
 C H 418 419
 Bethke R M Record P R
 Kirk C H and Kennard
 D C 418
 B thke R M see Bohstedt G
 431
 Bettis E S 600
 Beissonoff N 80 320
 Beissonoff N nd Stoett F
 336
 Beissonoff N and Vertruyen
 H 322

- Bicknell I 464
 Bielg H J see Kuhn R 39
 43 52
 Bierich R and Languzzi A
 160
 Bilger F see Sobotka M 366
 Bills C E 362 363 365 381
 386 404 405 415 419 594
 597
 Bills C E and Brickwedde
 F G 370 371
 Bills C E and Honeywell F
 M 348
 Bills C E Honeywell E M
 and Cox W M 370 371 380
 Bills C L and McDonald
 F G 409
 Bills C E McDonald F G
 and Spies T D 240
 Bills C E Massengale O N
 Hickman A C D and Gray
 E L 344 401
 Bills C E Massengale O N
 and Imboden M 344
 Bills C I Massengale O N
 Imboden M and Hall H
 384 411
 Bills C E and Wierck A M
 419
 Bills C I see Massengale
 O N 306 415 416
 Binkley S B Cheney L C
 Holcomb W F McKee
 R W Thayer S A Mac
 Corquodale D W and
 Doisy E A 482 487 493
 Binkley S B MacCorquodale
 D W Cheney L C Thayer
 S A McKee R W and
 Doisy E A 484 486 489
 499
 Binkley S B MacCorquodale
 D W Thayer S A and
 Doisy E A 484
 Binkley S B McKee R W
 Thayer S A and Doisy
 E A 482 485 488-490
 Binkley S B see Doisy L A
 494 499
 Binkley S B see MacCor-
 quodale D W 487 493
 Binkley S B see McKee F
 W 487 484 486 487 496
 Binkley S B see Richtert D
 494 499
 Binkley S B see Thayer S A
 507 503
 Bing F C see McCay C M
 197
 Birch T W 213
 Birch T W and Gyorgy P
 198 199 471 472
 Birch T W Gyorgy P and
 Harris S J 213
 Birch T W and Harris L J
 113 131 147
 Birch T W Harris L J and
 Ray S 318
 Birch T W and Mapson L
 W 123
 Bird H R see Kline O L
 522
 Bird H R see Oleson J J
 214 234 269 477
 Bird O D see Thayer S A
 503
 Birkhäuser H see Sullmann
 H 147 143
 Birkofer L see Weygand F
 172
 Bishop K S see Evans H M
 437 436
 Biskind G R see Gluck D
 326
 Bjälfe G see Nilsson R
 475
 Bjorksten J 580
 Black A 563
 Black A see Greene R D
 107 156
 Black A see Holaday H A
 567
 Black A see Steenbock H
 343 404 414 415 421
 Black S see McKibbin J M
 214 270
 Blackfan A D and Wolbach
 S B 95
 Blackfan A D see May C D
 56 93 94
 Blanchard E see Jeans P C
 92
 Blanchard K C 284
 Blanchpain C P see Meunier
 P 129
 Blankenhorn M A and Spies
 T D 246
 Blaso J G 564
 Bleyer B and Kallman O
 154
 Bleyer B see Schlemmer F
 295 396
 Blek M S C see Niekerk J
 van 419
 Block R J and Cowgill G R
 575
 Bloor W R see Sider R H
 438
 Blumberg H 458 467 544
 Blumberg H and Grady H G
 544
 Blumberg H and McCollum
 E V 544
 Blumenfeld C M 400
 Blumer B see Schopfer W H
 455
 Blyth A W 154
 Boas M A 469 473 476
 Bock F and Wetter F 346
 Bock F see Windaus A 344
 403
 Bodansky A and Jaffe H L
 474
 Bodansky O see Lewis J M
 8
 Bodendorf K 560
 Boer A G Niekerk J an
 Reerink E H and Wijk A
 van 346 349 359 407 595
 Boer A G Reerink E H
 Wijk A van and Niekerk
 J van 344 349 362
 Bohne A see Kropp W 555
 560
 Bohstedt G Bethke R M
 Edgington B H and Robin-
 son W L 431
 Bohstedt G see Phillips P H
 96
 Bohstedt G see Rupel I W
 432
 Boissevain C H Drea W F
 and Schultz H W 185
 Boland E W see Snell A M
 507
 Bolin D W see Williams
 J K 96
 Bollen W B see McBurney
 C H 255 270
 Bollman J L see Butt H R
 508
 Bollman J L see Snell A M
 507
 Bomskov C see Rominger I
 479
 Bonner D see Bolet J
 373
 Bonner J 186 219 41
 Bonner J and Axman C
 267
 Bonner J and Bonner D
 323
 Bonner J and Buchman E R
 119
 Bonner J and Greene J
 133
 Bonner J see Addcott F T
 243
 Bonner J see Went F W
 133
 Bongsnoore A see Martin F
 30
 Bonstedt K 3
 Booher I L Ju 106 19 573
 610
 Booher I L and Porter M B
 86
 Booher L L and Work J T
 580
 Booth A N see Wegner M I
 120 194 216 271 478
 Booth R C 18
 Booth V H 176
 Bord M see Park E A
 336
 Borgeaud P see Windau A
 357
 Borson H J and Mettler B R
 14
 Borson H J see Stockholm
 M 131
 Borsook H 29

AUTHOR INDEX

- Borsook H Davenport H W
 Jeffreys C E P and Warner
 R C 295 98 327
 Borsook H and Dubnoff
 J W 543
 Bosshard R and Hefti F 576
 Botcharsky S 556
 Boulton C G V 561
 Boulanger P see Kuhn R
 180 181 193
 Bourdillon R B Bruce H M
 Fschmann C and Webster
 T A 41
 Bourdillon R B see Angus
 T C 370
 Bourdillon R B see Ask w
 T A 363 383
 Bourdillon R B see Webster
 T A 378 370 371
 Bourne G 3 2 334
 Bourne G and Allen R 374
 330
 Bourquin A and Sherman
 H C 183 241 269 477
 Bowthorn P W see Stee hock
 H 33
 Bowdell D see Meunier P 503
 509
 Bowet D see Tréfont J 0
 Bowden P P Mort S S D D
 and Sn w C P 81
 Bowden P P and Snow C P
 30
 Bowen D M see Feser I F
 490 497
 Bowen W S 554
 Bowers R E and McCay
 C M 4 95
 Bowman K M see Jolliffe N
 46
 Boyce E P and McFetridge
 E M 68
 Boyd K see Daum K 333
 Boyd W L see Gullken T
 W 432
 Bracnott 343
 Bragg J H 509
 Braileo o H e Rall P P
 47
 Brauchi I 605
 Brauchli F see Häusler L F
 367 368 413
 Bauer L and Seal H 605
 Braun J v and Rudolph H
 65
 Bray W F and Kelly O R
 508
 Bray W F see Kelly O R
 568
 Brater J G and Curtis A C
 55
 Brazier D see Cerver J 5
 470
 Brederick H 170
 Brederick H Kötting M
 and Berger E 170
 Breeze B B see Claesen D W
 89
 Bresch H 563
 Brewer W see Todhunter
 E N 516
 Brickwedde P G see Bll
 C E 370 371
 Briggs A P see Sydenstrcker
 U P 477
 Briggs G M see Hegsted D
 M 477 5 541
 Brinch U Spehr H and
 Sander C E 558
 Brinkhaus K M Smith H P
 and Warner F D 507
 Brinkhaus K M and Warner
 I D 462
 Brinkhaus K M see Hawkin
 W B 309
 Brinkhaus K M see Scanlon
 G H 506 507
 Brinkhaus K M see Smitl
 H P 503
 Brinkhaus K M see Warner
 I D 48 50 507 09
 Brinto H 564
 Brod A F and Last B R
 563 565 605
 Brockelby H N 566
 Brockmann H 39 344 370
 378 381 397 387 388 390
 410 605
 Brockmann H and Busse A
 344 383 387-389 400
 Brockmann H and Chen
 Y H 413
 Brockmann H and Tecklen
 burg M L 50 78 79
 Brockmann H see Kuhn R
 38-41 44 53 74 75 80 90
 Brockmühl M and Ba tholo-
 maus E 610
 Brod W R see Mowry D T
 535
 Broekmeyer J see Brugh A A
 H vanden 38
 Bröm l H e Negelein P
 175 184
 Brooks G 180
 Brosteaux J e Green D J
 28 234
 Brown A see Drake T C H
 410
 Brown F F see Goettisch M
 458
 Brown I F see Gray E I
 86
 Brown G B e Vigneud V
 du 543 54 546
 Brown H B Shohl A T
 Chapman I F Rose C S
 and Sauerwein I M 414
 41
 Brown H B see Shohl A T
 470
 Brown J B and Frankel J
 534
 Brown J B and Stoner G C
 534
 Brown J B see Mowry D T
 535
 Brown J B see Shnowaro
 G 533-535
 Brown R A see Emmett
 A D 170
 Brown R A see Thayer S A
 503
 Brown W R see Burr G O
 467
 Browne C A see Nelson E K
 317
 Browne I A see Plot M M
 412
 Bruce H M see Angus T C
 370
 Bruce H M see Ake w F A
 383
 Bruce H M see Bourdillon
 R B 41
 Bruckner V and Sant
 Cy rky A 14
 Brüll W Sah i i T
 411
 Bruin H R Oshoff J and
 Wolff I K 69
 Brun G 387
 Brunken J s Windau A
 3 f
 Bruan v 7
 Brunner O and Baron L
 150
 Buchan n J M see Hastings
 A B 42
 Buchanan K s see Sure H
 89 145
 Buchholz K 34
 Buchholz K see W idaus A
 349 363 374 407
 Buchman I R 110 111 576-
 578
 Buchman E R and Williams
 R R 16
 Buchman I R Williams R
 R and Reszke y J C 111
 Buchman I R see Bonner J
 110
 Buchman I R see Williams
 R R 104 10
 Buckland H see Hoyle J C
 380
 Büchi J see Karrer P 33
 38
 Büeding F and Worts H
 148
 Buil M V see Steenbock H
 53
 Buhr T and Schoenenberger
 W 570
 Bumbalo T S see J it r W
 W 330 333
 Bunga v 9
 Bunker J W M Harris R S
 and Mosh R L M 369
 Bunker J W M see Harris
 R S 368
 Burway A see Ditty J W
 70

- Burgeff H 134
 Burk D Wenzler R J and Vigneaud V du 475
 Burk D see Allison F T 469 474 475
 Burk D see Gyorgy P 470 471
 Burk R F see Beard H H 369
 Burkart W see Reichel I 176
 Burke J C and McIntyre A R 14
 Burkhardt C N see Alder ley J B 402
 Burr G O Brown W R and Mosely R L 462
 Burr G O and Burr M M 531 537
 Burr G O see Evans H M 436 437 454 456 461 462
 Burr G O see Hansen A F 539
 Burr M M see Burr C O 531 537
 Burr W P see Davidson F S 553
 Burrows G see Fawcett I W 568
 Burström D see Euler H v 320
 Burstrom D see Nilsson R 475
 Bushill J H see Lampitt L H 565
 Busing K H and Peters F 325
 Busse A see Brockmann H 344 383 387-389 400
 Busse P 397
 Busse P see Windaus A 380
 Bussmann G see Karrer P 449
 Butenandt A Hausmann I Paland J Dresler D von and Meinerts U 407 408
 Butler A M see Mindlin R L 318 319 330
 Butler R F see Sebrell W H 191 193
 Butt H R 504
 Butt H R and Snell A M 481 494 508 509
 Butt H R Snell A M and Osterberg A E 482 504
 Butt H R Snell A M Osterberg A F and Bollman J I 506
 Butt H R see Clark R I 504 507
 Butt H R see Bell A M 507
 Button L L see Hawley F F 336
 Buxton L O 564 565
 Buxton L O and Colman H H 564
 Buxton L O and Simons L J 564 565
 Buy H G du see Wald G 84
 Byers D J see Koelsch C F 497
- C
- Caccia P 573
 Cahn F J and Harris B R 557
 Cahnmann H see Schlemmer F 303 306
 Cailleau R 32
 Cake W F 304
 Calder D C see Ferguson L K 507
 Callow R K 365 368
 Callow R K see Angus T C 370
 Callow R K see A Kew P A 389
 Campbell K see Holmes H N 330 334
 Campbell W P see Fieser I F 491 494 496 497 499 501
 Campion J F Henry K M Kon S K and Mackintosh J 403 406
 Camps R 524 2
 Campsie H G 595
 Cannon M D and Emmerson G A 596
 Capper N S McKibbin I M W and Prentice J H 50
 Card I F 461 463
 Carlén R B see Smith L I 447
 Carlén H see Ohle H 314
 Carlson L A see Rivers A B 334
 Carlson W A 595
 Carlsson F V and Sherman R C 183 192
 Carpenter A D see Barnes D J 49
 Carr I H 565
 Carr I H and Jewell W J 51 568
 Carr I H and Price I A 53 78 80
 Carrington H C see A H R G 591 308
 Carruthers C 597
 Carter C W Kersley H W and Peters R A 500
 Carter C W and O'Brien I R 214 219 503 501 502
 Carter H I and Melville D B 50
 Carter, H I and Ney L I 501
 Carvajal Torero J de see Martin G J 210 549 579
 Carven R 501
 Carver J S Robertson I I Brazier D Johnson R H and St John J L 49
 Casal 50
 Case L M 148
 Casady H G see Rider T H 369
 Castle D C Gillam A I Heilbron I M and Thompson H W 63
 Cavallini C see Bachstez M 320
 Cawley J D see Gray E I 70 8
 Centanni F 509
 Cerecedo I R 575
 Cerecedo L R and Hennesy D J 118
 Cerecedo I I and Kaszuba I J 118
 Cerecedo I R and Thornton J J 118
 Chabre I see Chevallier A 80
 Chaikoff I I and Connor C I 44
 Chaikoff I I see Intenma C 48
 Chaikoff I I see Perlman I 46
 Chaix P and Fromageot C 549
 Chakravorty P N see Fernholz E 3
 Chalkley H W see Voegtlin C 549
 Chandler J P see Vigneaud V du 517 518 517
 Chandler I A see Derby G S 93
 Chaney H K 597
 Channon H J Loach J V and Trim G R 518
 Channon H J Manfold M C and Platt A P 540
 Channon H J Pitt A P and Smith J A B 546
 Channon H J and Smith J A B 546
 Channon H J see Aylward I I 57 46
 Channon H J see Beeton A W 4
 Channon H J see Drimmon I J C 61
 Chapman I E see Brown H F 414 421
 Chant A J and Khaustov A W 189
 Chase A M 180
 Chase A M see Hecht S 89
 Chase D see Taylor F 330
 Chase I I 131 132
 Chen Y H see Brockmann H 413
 Cheney G 50
 Cheney I C see Binkley S B 489 484 486 487 489 493 499
 Cheney L C see MacCorquodale D W 493
 Cheley I I see Rolando H F 564

- Chesney J and McCoord A
 B 95
 Chesney J W D 596 605
 Chevallier A 8
 Chevallier A and co-workers
 85
 Chevallier A and Chabre P
 80
 Chevallier A and Chorony Y
 94 316
 Chevallier A Chorony Y and
 Matheron R 94
 Chevallier A and Doubouloz
 P 80
 Chick H 197
 Chick H Copping A M and
 Roscoe M H 197
 Chick H El Sadr M M and
 Worden A N 198 199 214
 Chick H Macrae T F Mar
 tin A J P and Martin C J
 214 250
 Chick H and Roscoe M H
 102 137 156
 Chick H Worden A N and
 El Sadr M M 214
 Chick H see Hume E M 61
 81
 Chidow D 553
 Chon H and Farmer C J
 326
 Chutenden R H and Under
 hill F P 220
 Cholnoky L see Zechmeister
 L 41 46 51
 Chorony Y see Chevallier A
 94 316
 Choy F see Rinehart J F
 334
 Chitt R E see Fuson R C
 85
 Christensen C E see Ole
 fabrik Aarhus 565
 Christensen E 413
 Christensen K 545
 Christian W see Warburg O
 154 172 174 175 179 184
 185 270 228 230-233 235
 238 586
 Christ N A von and Anger
 V 367 368
 Christensen N W G 591
 Christensen W G Jones W
 S and Moness E 562
 Christensen W G and Mo
 ness E 562
 Chuang C K 350
 Chubb A E see Norris E R
 79 428
 Cimmino A 297
 Claiborne T S see Hurxthal
 L M 380
 Claus I 484 608
 Clark A B see Wold G 90
 Clark J 405
 Clark L M see Mills W H
 114
 Clark R L Dixon C F Butt
 H R and Snell A M 504
 507
 Clarke T W F 564
 Clark W M 209
 Clarke H T and Behr L D
 267
 Clarke H T and Gurin S
 110 111 113
 Clarke H T see Darby H H
 404
 Clausen S W 85 89
 Clausen S W Baum W S
 McCoord A B Ryden J O
 and Breese B B 89
 Clausen S W Breese B B
 Baum W S McCoord A B
 and Ryden J O 89
 Clausen S W and McCoord
 A B 94
 Cleckley H M see Kruse
 H D 191
 Clift F P and Cook R R
 148
 Cline J K 611
 Cline J K Williams R R and
 Finkelstein J 103 107 116
 Cline J K Williams R R
 Ruehle A E and Water
 man R E 105 109
 Cline J K see Williams R R
 100 103 116 126
 Cloetta A 276
 Clutton R F Schoenheimer
 R and Rittenberg D 541
 Cobenzl A see Skraup Z H
 224
 Cohen F H 182
 Cohen I see Vichoever A
 465 465
 Cohen J Y see Zuckerman
 I C 507
 Cohen P P see Krebs H A
 228
 Cohn B N E see Jones J H
 475
 Cohn M see Vigneaud V du
 543 545 546
 Cohn W F and Greenberg
 D M 474
 Colman H B see Buxton L
 O 564
 Colman J see Gabriel S 206
 Conant J B Cramer R D
 Hastings A B Klempner
 F W Solomon A K and
 Vennesland B 442
 Conger T W and Elvehjem
 C A 211
 Conklin R E see Matill H A
 436
 Connell S J B and Zilva S S
 290
 Connolly A G 18
 Connor C L 38
 Connor C L see Chaikoff I
 L 544
 Connor R see Floyd L W
 304
 Conover F H 478
 Cook A H see Kuhn R 114
 Cook B B see Morgan A F
 203
 Cook E F 130 13
 Cook E W see Major R T
 590 591
 Cook R R see Clift F P
 148
 Cooley T B see Slyker F
 40
 Coombes A I Ott G L and
 W sneaky W 55
 Cooper E A 521
 Copen P A and Metz G A
 385
 Copping A M 198 212 404
 Copping A M and Koren
 chevsky V 48 462
 Copping A M see Bergel F
 445
 Copping A M see Chick H
 197
 Corbett A 590
 Corbett R E see Holmes H
 N 3 60-6 72 75 81
 Cordus Euricius 290
 Corlette N B see Youmans
 J B 97
 Cornea F E 330
 Cornbleet T 334 539
 Corran H S Dewan J G
 Gordon A H and Green D
 E 175
 Corran H S and Green D F
 175
 Corran H S Green D C and
 Straub F B 173 175
 Corran H S see Green D C
 175
 Corran H S see Straub I B
 173
 Correll J T 409 420
 Cortis Jones B see Lemberg
 R 328
 Coulson E A 490
 Coward K H Dyer F J
 Morton R A and Gaddum
 J H 79 80
 Coward K H Key K M and
 Morgan G E 413 420
 Coward K H see Drummond
 J C 61
 Coward K H see Knapp A
 W 385
 Coward K H see Mead T H
 81
 Coward K H see Underhill
 S W F 54 75
 Cowgill G R 13
 Cowgill G R and Palmeri
 M L 145
 Cowgill G R see Block R J
 575
 Cowgill G R see Himwich
 H E 145

- Cowgill G R see Street H R 188 191
 Cowgill W W 557
 Cox E G 294
 Cox F G and Goodwin T H 291 294
 Cox E C and Hirst I L 294
 Cox E C Hirst L L and Reynolds R J W 297
 Cox W M see Barton R W 556
 Cox W M see Bills C E 370 371 380
 Cragwall G O see Pasternack R 589
 Cramer R D and Kistia kowsky G B 542
 Cramer R D see Conant J B 542
 Crandall L A see Robinson H E 264
 Cravens W W see Holmes C E 463
 Creed R H see Morton R A 70
 Cregor N M see Hoffman C 573
 Creighton M M see Anderson R J 278
 Criegée 354
 Crumm P D and Short D M 98
 Cristallo A G 587
 Crook E M and Hopkins F G 327
 Cross R J 569
 Cuenod B 557
 Currie D W 463 465
 Curtis A C see Braser J G 55
 Cuthbertson W F J Ridge way R R and Drummond J C. 437 451 452 456
- D
- Dack G M 524
 Daft F S Frazer H F Sebrell W H and Pittman M 246
 Daft F S and Sebrell W H 269
 Daft F S Sebrell W H Babcock S H and Jukes T H 269 271
 Daft F S Sebrell W H and Lillie R D 544
 Daft F S see Lillie R D 544
 Dainow I 330
 D'Alelio G F 59
 Dalldorf G 333 336
 Dalmer O and Heyns K 307 589 590
 Dalmer O and Molt T 314
 Dalmer O and Werder F v 605
 Dalmer O Werder I v and Molt T 387
 Dalmer O and Wieters H 587
 Dalmer O see John W 603
 Dalyell I J see Farli 477
 Dam H 481 484 500 507 509
 Dam H Ceiger A Glavind J Karrer P Karrer W Rothschild E and Salomon H 487 505
 Dam H and Glavind J 467 463 482 483 494 502 504 507 509
 Dam H Glavind J and Karrer P 496 497 503
 Dam H Glavind J Lewis L and Tage Hansen E 506
 Dam H Glavind J Orla Jensen S and Orla Jensen A D 494
 Dam H Karrer P and co worker 482
 Dam H and Lewis L 483
 Dam H and Schønheyder F 481 483
 Dam H Schønheyder F and co-worker 482
 Dam H Schønheyder F and Tage Hansen F 482 506 507 509
 Daniel E P and Munsell H E 16
 Daniels F see Hoffman R M 371
 Daniels F see Kon S K 362 368 409
 Dann F P 504
 Dann W J 65 86 221 234
 Dann W J and Evelyn K A 78
 Dann W J and Kohn H I 250
 Dann W J see Kohn H I 244
 Dannenberg S J 575
 Darby H H and Clarke H T 404
 Darby W J see Day P L 191 524
 Darby W J see Langston W C 524
 Das N B 298
 Das N B see Adler E 228
 Das N B see Bentsath A 516
 Das N B see Günther G 928
 Das N see Guha B C 279
 Dass B 554
 Daum K Boyd L and Paul W D 333
 Dauvergne M see Mouri quard G 326 37
 Davenport H W see Borsook H 295 298 327
 Davidson F S and Burra W P 553
 Davidson S see Ralli E P 56
 Davies A W and Moore T 86 91
 Davis F P see Kahler H 189
 Davis H J Norris L C and Heuer G F 191 194
 Davi J C and McClemon J 374
 Davis M see McCollum B V 12 37 57
 Davis O L see Lingane J J 183
 Davison H G see Morgan A F 253
 Day C D M see Taylor G F 428
 Day H G see Shils M 148
 Day P L and Darby W J 191
 Day P L Darby W J and Langston W C 191
 Day P L and Langston W C 183
 Day P L Langston W C and Darby W J 524
 Day P L Langston W C Darby W J Wahlun J G and Nims V 574
 Day P L Langston W C and O'Brien C S 191
 Day P L see Langston W C 524
 Day P L see Totter J R 541
 De N K 82
 Decker C T 518
 Dedrick B W 573
 Deeson W M see Baumann C A 86
 Deffner M see Franke W 177
 Dehn F B 592
 Dells B see Shettles L B 505
 Deloson A M see Langlois G A 536
 DeMasters C V see Abbott O D 545 548
 Demole V 193 465
 Demole V Isler O Ringier B H Salomon H and Karrer P 449
 Demole V see Euler H v 53 75
 Demole V see Reichstein T 314
 Denel H J see Harper H A 148
 Denis W see Folin O 207 210
 Dennison R 186 324
 Deppe M see Reichel S v 393
 Deppe M see Windaus A. 372 374 376 379 383 400
 Derby G b Chandler P A and Sloan L L 93
 Desnuelle P see Kuhn R 6 172
 Deuel R E see Rahn O 324

- DeVaney G M Munsell H E and Titus H W 406 419
 DeVaugh N M see Syden stricker V P 477
 DeVries T see Moore R B 371
 Dewan J G 228
 Dewan J G and Green D E 173 174 2 9
 Dewan J G see Corran H S 175
 Dewan J G see Green D P 173 228
 Dewar M M see Hamilton B 409
 Dicken D M see Landy M 475
 Dickens C 556
 Dickens F 228
 Diehl F 330
 Diels O and Karsten A 352
 Demair W 403
 Diets C F 8 605
 Dietzel E see John W 441 443 449 457
 Dignonet L see Lwoff A 246
 Diley W E see McCay C M 197
 Dimck M K and Lepp A 269
 Dimick M K and Schreffler C B 211 214 216
 Diamond N S 284
 Dimroth K 375 399 402
 Dimroth K and Johnson H 402
 Dimroth K. and Paland J 349 364 407 408
 Dimroth K see Paland J 349
 Dimroth K see Windaus A 399
 Dithmar K. see Windaus A. 375 391
 Dittmar J see Klen K E 532
 Dixon C F see Clark R L 504 507
 Dixon M 176 228
 Dixon M and Lutwak Mann C. 228
 Dixon M and Zerfas L G 227
 Dixon W E and Hoyle J C 380
 Dossy E A 48?
 Dossy E A and co-workers 482
 Dossy E A. MacCorquodale D W Thayer S A Binkley S B and McKee R W 494 499
 Dossy E A. see Binkley S B 48? 484-490 493 499
 Dossy E A. see MacCorquodale D W 487 493
 Dossy E A see McKee R W 482 484 486 489 496
 Dossy E A. see Richtert D 494 499
 Dossy F A see Thayer S A 50?-504
 Dolliver M A see Ansbacher S 494 499
 Dols M J L Jansen B C P Szoo G J and Maas G J vander 423
 Domagk G ?
 Donath W F see Janten B C P 100 102
 Dondroff M 185
 Dorcas J see Supplee G C 599
 Dorfman A Horwitz M K Koser S A and Saunders F 249
 Dorfman A Koser S A Reames H R Swingle K F and Saunders F 246
 Dorfman A see Koser S A 43
 Dorfman A see Saunders F 246
 Dornow A 17
 Dornow A see Baumgarten P 127
 Dorp W A van see Hooge werf S 224 26?
 Doubovols P see Chevallier A 80
 Doudoroff M 193
 Dra bach F 556
 Drake T G H Tisdall F F and Brown A 410
 Dre W F see Boissevain C H 185
 Dresler D von see Butenandt A 407 408
 Dressel J W 573
 Dryfus A see Meunier P 503 509
 Drigalski W v 84
 Drill V A 190
 Drift V A and Sherwood C. R 145
 Drumel G and Hubert L 471
 Drummond J C. 13 259 459
 Drummond J C Bell M E and Palmer E T 85
 Drummond J C Channon H J and Coward K H 61
 Drummond J C. and Funk C 2 0
 Drummond J C and Günther E R 404
 Drummond J C and Hoover A A 433
 Drummond J C. Noble R. L. and Wright M D 460
 Drummond J C Singer E and MacWalter R. J 438 440
 Drummond J C see Cuthbertson W F J 437 431 45? 456
 Drummond J C see G Ham A F ?9
 Drummond J C see Guba B C. 102
 Drummond J C see Haslewood G A D 344 387 388
 Drummond J C see Hassan A 16
 Drummond J C see Heifbron I M 60 61 63
 Drummond J C. see Moss A R 437 438
 Drummond J C see Narayana B T 155
 Drummond J C see Rea J L 54 56
 Drummond J C see Rosenheim O 80
 Drummond J C see Wright M D 449 457
 Dubin H E see Funk C 562
 Duboff J W see Borsook H 643
 Dubos G 560
 Dufat R see Massart L 14? 143
 Duffan R see Lecoq R 409
 Dunn J T see Fieser L. F 497
 Dutcher R A see Bechdel S I 101 150
 Dutcher R A see Knight C A. ?02
 Dutt A see Acton H W 130
 Dyer F J 415
 Dyer F J see Coward K H 79 80
- E
- Eakin R E McKinley W A and Williams R J 476
 Eakin R E Snell E E and Williams R J 476
 Eakin R E and Williams R J 212
 Eakin R. E see Pennington D 476
 Eakin R. E see Snell E E 474 475
 East B R. see Brod A E 563 565 605
 Eastcott E V ?75 470
 Ebbara T see Fujita A. ? 319 3 0
 Eck J C and Thomas B H 409
 Eck J C Thomas B H., and Yoder L 409
 Eckardt R E and Johnson L V 189 191
 Ecker E E Pillemer L. Griffiths J J and Schwartz W P 331
 Ecker E E Pillemer L. Wertheimer D and Gradia, H 331
 Eckhardt H J 360

- Eckhardt H J see Windaus
 A 361 362
 Eckhardt R E see György P
 197 211 544
 Eckstein H C 538
 Eckstein H C see Tucker H
 F 543
 Eddy W H see Kohman E F
 312
 Eddy W H see Williams
 R R 521
 Edel V see Mouriquand G
 326 337
 Edgar C L Fl Sadr M M
 and Macrae T F 198 211
 214 266 269
 Edgar C E and Macrae T F
 197 253 266
 Edgar C E see Macrae T F
 522
 Edgington B H see Bohstedt
 G 431
 Edie C S Evans W H
 Moore B Simpson G C E
 and Webster A 99
 Edisbury J R 78 81
 Edisbury J R Gillam A F
 Heilbron I M and Morton
 R A 62
 Edisbury J R Morton R A
 and Simpkins G W 78
 Edisbury J R Morton R A
 Simpkins G W and Lovern
 J A 58 71
 Edisbury J R see Lederer E
 37
 Edisbury J R see Pritchard
 H 63 72
 Edmund C see Friderichsen
 C 93
 Eekelen M van 317
 Eekelen M van Emmerie
 A Julius H W and Wolff
 K L 79
 Eekelen M van and Heine
 mann M 325 376
 Eekelen M van see Emmerie
 A 187 317 413
 Eggleston L V see Krebs
 H A 130-139 141
 Eggleston W G F 123 142
 Eichelbaum C 583
 Eicher B L 554
 Eijkman C 9 100
 Einarson L and Ringstead A
 462 464
 Eisenbrand J and Lienz M
 591
 Eisenbrand J and Picher H
 583
 Ekblad M and Wohlfart G
 462
 Elderkin J K and Hofman
 E 599
 Elger F 588 589 591
 Eliot M M Nelson E M
 Barnes D J Browne F A
 and Jense R M 412
 Eliot M M Southern S P
 Anderson B A and Arnim
 S 427
 Elias O A 600
 Elley H W and Waddell J
 599
 Ellinger A and Henzel M
 285
 Ellinger G F see Goldsmith
 G A 335
 Ellinger P and Koshara W
 154 156 188
 Elliott M C Isaacs B and
 Ivy A C 503 504
 Ellis L N and Zmachinsky A
 190
 Ellis N R and Isbell H S
 538
 Ellis N R see Spadola J M
 538
 Ellman P see Abbasy M A
 334
 Elmby A and Warburg E
 517
 Elmby A and Wirth T 335
 Elphick G K see Key K M
 323
 El Ridi M S see Gillam A E
 60 71
 El Sadr M M 191
 El Sadr M M Hind H G
 Macrae T F Work C E
 Iythgoe B and Todd A
 R 264
 El Sadr M M Macrae T F
 and Work C E 183 197
 El Sadr M M see Chick H
 198 199 214
 El Sadr M M see Edgar C E
 198 211 214 266 269
 Eldsen S R 137 139
 Elvehjem C A 469
 Elvehjem C A and Koehn
 C J 156 203 254
 Elvehjem C A Koehn C J
 and Oleson J J 469
 Elvehjem C A Madden R J
 Strong F M and Woolley
 D W 220 242 250
 Elvehjem C A Sherman
 W C and Arnold A 101
 Elvehjem C A see Anderson
 H D 462
 Elvehjem C A see Arnold A
 131 541
 Elvehjem C A see Axelrod
 A E 188 192 234 244 249
 Elvehjem C A see Conger
 T W 211
 Elvehjem C A see Frost D
 V 250
 Elvehjem C A see Hegsted
 D M 214 216 474 477
 527 541 542 545
 Elvehjem C A see Johnson
 B C 526
 Elvehjem C A see Keenan
 J A 521
 Elvehjem C A see Kline O L
 521 529
 Elvehjem C A see Koehn
 C J 247 253
 Elvehjem C A see Kohler
 G O 526
 Elvehjem C A see Lipschitz
 M A 124
 Elvehjem C A see Lipton
 M A 120
 Elvehjem C A see McKibbin
 J M 214 70
 Elvehjem C A see Mannering
 G J 188
 Elvehjem C A see Mickelsen
 O 53 271
 Elvehjem C A see Nielsen
 E 477
 Elvehjem C A see Oleson
 J J 214 254 269 477
 Elvehjem C A see Sober H
 A 138
 Elvehjem C A see Steenbock
 H 385
 Elvehjem C A see Wagner
 J R 183
 Elvehjem C A see Waisman
 H A 240 205
 Elvehjem C A see Wegner
 M I 150 194 216 50 271
 478
 Elvehjem C A see Woessner
 W W 317 318
 Elvehjem C A see Woolley
 D W 239 253 255 257 208
 260 264 270
 Embree N D 61 63
 Emde H 01
 Emmerie A 79 155 183 187
 317 320 321 402 403
 Emmerie A and Eekelen M
 van 187 317 413
 Emmerie A and Engel C
 402 453 456 464
 Emmerie A see Eekelen M
 van 79
 Emerson G A and Evans
 H M 214
 Emerson G A Mohammad
 A Emerson O H and
 Evans H M 199
 Emerson G A see Cannon
 M D 526
 Emerson G A see Emerson
 O H 437 439
 Emerson G A see Evans H
 M 430 437 438 449 451
 Emerson G A see Shimotori
 N 462
 Emerson O H 443
 Emerson O H Emerson G A
 and Evans H M 437 439
 Emerson O H and Smith
 L I 436 444
 Emerson O H see Emerson
 G A 199
 Emerson O H see Evans
 H M 435 437 438 449 451

- Emerson O H see Ottcott
H 5 440
- Emmett A D and McKin
L H 154 571
- Emmett A D Peacock G
and Brown R A 179
- Emmett A D see Thayer S
A 503
- Emte W see John W 449
457
- Endler F 387 463
- Engel C see Emmerie A 40
453 456 464
- Engel R 507
- Engel R W 913 268
- Engel R W and Salmon W
D 545
- Engel R W see Philip P H
191 69
- Engel V H Westlund J and
Schenck R T 591
- Engler C 225
- Entenman C and Chakoff
I L 548
- Eoff J R 553
- Epprecht A see Karrer P
496
- Epstein I M see Farmer
C J 335
- Epstein R see Karrer P 239
- Erbach H see Ohle H 314
- Erdelyi J see Roenthal J
79 80
- Erdmann E and Bedford F
534
- Ericksen T S see Euler H v
228 233
- Ernotte M 561
- Escher H H see Westlatter
R 39 43
- Escher R see Karrer P 442
444 450 4 3
- Espil L and Genevo L 3 2
336
- Euler B v Euler H v and
Karrer P 53
- Euler H v 14 37 57 157 18
2 9 230 235
- Euler H v and Adler E
154 155 174 18 189 230
235 237
- Euler H v Adler P and
Ericksen S 2 8 233
- Euler H v Adler F and
Günther G 2 8
- Euler H v Adler E Günther
G and Das N B 3
- Euler H v Adler E and
Hilström H 174 228 230
236
- Euler H v and Ahlström L
88
- Euler H v Albers H and
Schlenk F 270 230 234 585
- Euler H v and Bauer F
37
- Euler H v and Burström D
320
- Euler H v Demok V
Karrer P and Walker O 57
75
- Euler H v Gard W and
Hilström H 38
- Euler H v and Cünther G
173 222
- Euler H v Günther G
Malmberg M and Karrer
P 76
- Euler H v and Hasse K
173
- Euler H v Herwinkcl H
and Schlenk F 222 236
- Euler H v and Hilström H
173
- Euler H v and Högberg B
145
- Euler H v and Karlson S
234
- Euler H v Karrer F Adler
E and Malmberg M 180
- Euler H v Karrer P and
Becker B 232
- Euler H v Karrer P Hell
ström H and Rydbom M
70
- Euler H v Karrer P
Klasmann T and Morf R
79
- Euler H v Karrer P and
Malmberg M 181
- Euler H v Karrer P Malm
berg H Schöpp K Benz
P Becker B and Fr P
167
- Euler H v Karrer P and
Solm sen U 70 77
- Euler H v Karrer P and
Walker O 70
- Euler H v Karrer I and
Zander P 331
- Euler H v Karrer P and
Zubrys A 54 72 7
- Euler H v and Klasmann
E 51 56 59 91 200
- Euler H v and Malmberg
M 20 320
- Euler H v Malmberg M
and Cünther C 230
- Euler H v and Mit C
90 317
- Euler H v and Myrbäck K
222 232 34
- Euler H v and Schlenk F
23 236
- Euler H v Schlenk F
Herwinkcl H and Högberg
B 230 33 235 240 244
- Euler H v Schlenk F
Meier L and Högberg B
241 222 241 250 57
- Euler H v Schlenk F and
Vestin R 232
- Euler H v and Schmidt G
83
- Euler H v Sode H and
Malmberg M 2 3
- Euler H v and Sven so T
3 9
- Euler H v and Vestin R
170 237 580
- Euler H v and Vrgu F
38
- Euler H v see Adler E 173
174 189 2 3 232
- Euler H v see Albers H
230
- Euler H v see Euler B v
53
- Euler H v see Karrer P
181 3 8
- Euler H v see Myrbäck K
230 231 236
- Euler H v see Schlenk F 23
- Evans E A and Stott L
137
- Evans H M 401
- Evans H M and Bhop K S
43 436
- Evans H M and Burr G O
436 437 404 406 461 462
- Evans H M Emerson O H
and Emerson G A 435 437
438 451
- Evans H M Emerson O H
Emerson G A Smith I I
Ugnade H E Prichard
W W Austin F L Hoehn
H H Opie J W and
Wawzonek S 449 451
- Evan H M Lepkovsky S
and Murphy E A 231
- Evans H M see Emerson G
A 199 214
- Evans H M see Emerson
O H 437 439
- Evans H M see Halliday N
199 211
- Evans H M see Lepkovsky S
S 156
- Evans H M see Shimotori N
467
- Evans W 337
- Evans W C Hendley W R
C and Happold F C
64
- Evans W H see Ide C S
99
- Evelyn K A 313
- Evelyn K A Malloy H T
and Rosen C 319
- Evelyn K A see Dann W J
78
- Eysenbach H see Fisher A G
177
- EWING D T Vandenberg J
M and Kamm O 485 486
503
- F
- Faght H J see Johnson B C
2 0
- Falk K F see Lewis J M
8

- Falke 333
 Farf Dalyell E J and Mackay 427
 Farmer C J and Abt A 335
 Farmer C J and Epstein I M 335
 Farmer C J see Abt A F 326 334 337
 Farmer C J see Chinn H 326
 Farmer E H and Van de Heuvel F A 536
 Farup P 557
 Faulkner J M and Taylor F H L 325 335
 Faulkner J M see Taylor F H L 335
 Faust W see Ott E 301 315 329
 Fawcett E W and Burrows G 563
 Fearon W R 80
 Feecey R E and Strong F M 184 192
 Feecey R E see Strong F M 184 187 192
 Feher G 603
 Fein H D see Jolliffe N 246
 Feldman J B 93
 Felsovary A v see Bernheim F 245
 Ferguson L K and Calder D G 507
 Ferguson W S 94
 Fernholz E 437 441 609
 Fernholz E Ansbacher S and MacPhillamy H B 496 503
 Fernholz E Ansbacher S and Moore M L 482
 Fernholz E and Chakravorty P N 352
 Fernholz E and Finkelstein J 451
 Fernholz E see Ahrens G 375
 Fernholz E see Ansbacher S 482 494 496 499 610
 Fernholz E see Windaus A 375 391
 Ferrari C G see Sherwood R C 606
 Feudenberg K 581
 Field H see Melnick D 124 129 148 240 241 248 249
 Fieser L F 482 484 493 496 497
 Fieser L F Bowen D M Campbell W P Fieser M Frey E M Jones R H Riegel B Schweitzer C E and Smith P G 496
 Fieser L F Bowen D M Campbell W P Frey E M and Gates M D 497
 Fieser L F Campbell W P and Frey E M 494 496 497 501
 Fieser L F Campbell W P Frey E M and Gates M D 491 496 499
 Fieser L F and Dunn J T 497
 Fieser L F and Frey F M 494 499
 Fieser L F Tishler M and Sampson W L 482 495-497 499 500
 Fieser L F Tishler M and Windler N L 496
 Fieser L F and Wieghard C W 497
 Fieser L F see Tishler M 450 493 496 497 499 500
 Fieser M see Fieser L F 496
 Fildes P 243 283 286
 Fildes P see Woods D D 283 285
 Filkitt S 176
 Fima O see Möller E F 209 211
 Fincke H 605
 Findlay C M and Stern R O 477
 Finkelstein J see Cline J K 103 107 116
 Finkelstein J see Fernholz E 451
 Finkelstein J see Major R T 262
 Finkelstein J see Stiller E T 255 257 259 261 264 271
 Finkelstein J see Williams R R 104 109
 Finland M see Strauss F 286
 Fischer E 262
 Fischer F G and Löwenberg K 488
 Fischer H and Landner F 378
 Fischer M see Berend N 335
 Fischer O 225
 Fischer W see Ruzicka L 75
 Fischermann C see Angus T C 370
 Fischer F 587
 Fischmann C P 422
 Fischmann C P see Bourdillon R B 415
 Fisher F G and Eysenbach H 177
 Fisher F G Roedig A and Rauch K 177
 Flanagan G E and Supplee G C 182 581
 Flanagan G E see Supplee G C 409 556 573 580
 Fleischmann W 458
 Fletcher W L 556
 Flexner L A see Gording R 222
 Flodquist L 594
 Floody R J see Knudson A 418
 Floyd L W Connor R and Adkins H 304
 Flynn J B see Scanlon G H 506 507
 Flynn J D see Smith H P 507
 Foder M E 405
 Fodor A see Abderhalden E 260
 Foldy Z and Gerecs A 576
 Folin O 370 464
 Folin O and Denis W 207 210
 Folkers K see Harris S A 204
 Folkers K see Keresztesy J C 198
 Folkers K see Stiller E T 255 257 261 264 271
 Follet D H see Tuymen F 572
 Folley S J and Kay H D 423
 Fontaine M see Guillemond A 186
 Forster 9
 Foster E G see Baumann C A 89
 Foster R H K Lee J and Solmsen U V 494 499
 Fouts P J Helmer O M and Lepkovsky S 214
 Fouts P J Helmer O M Lepkovsky S and Jukes T H 214 270
 Fouts P J Lepkovsky S Helmer O M and Jukes T H 254
 Fouts P J see Helmer O M 247
 Fraenkel Conrat H L see Todd A R 120
 Frandsen H 92
 Frank L see Léranth G 458
 Franke W and Deffner M 177
 Frankel J see Brown J B 534
 Franco F see Niekerk J van 407 409
 Frankenburger W see Zimmermann W 597
 Fraps G S and Kemmerer A R 53
 Fraps G S Kemmerer A R and Greenberg S M 53
 Fraps G S see Sherwood R M 96
 Fraps R M 598
 Fraser A H H see Auchl nachte D W 432
 Fraser R G J 567
 Frazer H F Topping N H and Isbell H 183 192 242
 Frazer H F see Daft F S 248

- Fraser H F see Pittman M 244
 Fraser J P see Hawley E E 336
 Frazier C N and Hu C K 92
 Frei P see Duler H v 167 181
 Frei P see Karrer P 165 177 179
 Freitag E 60?
 Freses A T 31
 Freudenberg E and György P 425
 Freudenberg E and Welker A 46
 Frey C N see Hess A F 418 419
 Frey C N see Kirby G W 556
 Frey C N and Light R F 594
 Frey C N see Light R F 419 426 573 594 598
 Frey E M see Feser L F 491 494 496 497 499 501
 Frey C N see Schultz A S 131 134 148 211 212
 Freytag R M see Morgan A F 430
 Fricker K 586
 Friedrichsen C and Edmund C 93
 Fridericia L S and Holm E 89
 Friedemann T E and Barborka C J 248
 Friedm u G J see Ralli E P 325
 Fries h. and Lohmann W 490
 Fries H see Fögl F 134 279
 Fritzsche H see Karrer P 157 160 163 181 233 238 43 437 44 444 445 449 450 453 457
 Frölich T see Holst A D 90
 Frohring W O see Barnett H M 569
 From V C Rowley C D and Lasky A W 601
 From geot C see Chaux P 549
 Frost D V and Elv hjem C A 250
 Fujita A and Ebihara T 302 319 370
 Fujita A lwatak D and M jata T 370
 Fulmer E I 469
 Fulmer E I see Lesh J B 78
 Funk C 10 10 100 270 340 561 573 587
 Funk C and Dubin H E 562
 Funk C. and Funk I C 445
 Funk C see Drummond J C 20
 Funk C see Sheets O 343
 Funk I C see Funk C 244
 Furter M and Meyer R E 453
 Fuson R C and Christ R E 65
- G
- Gabriel S and Colman J 206
 Gaddum J H see Coward k. H 79 80
 Gaede J see Windaus A 381
 Gáts Fichter M Reich H and Reichstein T 260 266
 Gáts Fichter M see Grüssner A 60 71
 Gal I 319
 Gams A and Locher F 594
 Gams A and Schreiber B 573
 Gander J and Niederberger W 336
 Gannon C F and McGovern T 317
 Gard U see Euler H v 38
 Garmier see Lwoff A 246
 Garratt D C 60 7
 Gastrock E A see Wells P A 304
 Gates M D see Fisher L F 491 496 497 499
 Gav n G and McHenry B W 280
 Gavin G see Longenecker H E 142
 Gav n G see McHenry B W 142 188 213 476
 Gav n N A 569
 Glicken H and Richter H 60
 Geig r A and Rosenberg A 144
 Geiger A see Dam H 48 505
 G eger A see Karrer P 69 70 76 449 474 481 483-485
 Getz F 557
 G evo s L see Ispl I. 3 336
 Gérald E 307
 Gerecs A see Foldi Z 576
 Gerischer W see Heglen F 28
 Germann A F O 563
 G rmann A F O see Barnett H M 569
 Gerstenberg R H J Ha t m n J I Russel G R and W l der T S 45
 Ghosh A R see Guha B C 319
 Ghosh B 331 336
 Ghosh B and Guha B C. 212 293 301
 Ghosh S see Acton, H W 130
 Gibb 210
 Gibbs H D 202
 Gerhake J 463
 Gllam A E 71
 Gllam A E and El Ridi M S 65 71
 Gllam A E Heilbron I M Jones W F and Lederer E 59 70
 Gllam A E Heilbron I M Lederer J and Rosanova V 68
 Gllam A F Heilbron I M Morton R A. and Drummond J C 79
 Gllam A E see Castle D C 63
 Gllam A I see Fdsbury J R 62
 Gllam A I see Heilbron I M 72 73
 Gillespie J M see Rubbo S D 83-286
 Gillies J see Smith A M 34
 Girard A see Sandulesco G 561
 Gir k. V 320 332
 Gir k. V and Krishnamurthy P V 37
 Giroud A 322
 Giroud A. and Leblond C P 330
 Glaban C see Trav s P M. 557
 Glanzmann E 403
 Glaser E 261
 Glavind J see D m H 462 463 482 483 494 496 497 50 -507 509
 Glick D 33
 Glick D and Antopol W 143
 Glick D and G R 36
 G l ksm nn t 611
 Glun H I 60
 Glynn H E s B ha a h A. L 437 454
 Goedecke F 587
 Goefdrb Y L 586
 Goese M A see McIlvaine S M 22
 Göthlin G F 336
 Goettisch M 436
 Goettisch M and Brown E F 48
 Goettisch M and Fappenheimer A M 431 444
 Goettisch M and R tzm nn J 469
 Goettisch M see Lappenheim A M 463
 Goldberg M W see Rucka L 70

- Goldberger J and Little R D
198 20 204 9 4 7
- Goldberger J and Tanner W
F 219
- Goldberger J and Wheeler
G A 220
- Goldberger J Wheeler G A
Lille R D and Rogers
L M 220 247
- Goldberger J Wheeler G A
Rogers L M and Seibell
W H 220
- Goldblatt H 371
- Goldblatt H and Soames K
M 418
- Goldblatt H see Beard H H
369
- Goldblatt H see György P
544 546
- Goldfarb W see Hmwech
H I 140
- Goldner J see Barron F S
G 139 141
- Goldman I see Akker P M
500 07
- Goldsmid G A and Fenger
G F 330
- Goldsohet G I 34
- Gonce J F see Anderson H
D 467
- Goode G P 598
- Goode G P see Rader T H
369
- Goodhart R S 149
- Goodhart R S and Sinclair
H M 130 149
- Goodwin T H see Cox F G
291 294
- Goodyear G H see Williams
R J 253 254 257
- Gordong R and Flexner L A
229
- Gordon A H Green D E
and Subrahmanyan V 176
- Gordon A H see Corran H S
170
- Gordon A H see Subrahman
yan V 176
- Gordon E S see Kmhle M
S 192 333
- Corkow R see Ahrens F B
224
- Coss H see McElroy L W
150 194 217 216 66 271
- Gottlieb H L see Quacken
bush F W 438
- Goudsmut J and Westenbrink
H G K 193 135
- Goudsmut J see Westenbrink
H G K 128 147
- Gourier A A 560
- Grab W 407 415
- Grads H see Ecker E E 331
- Grady H G see Blumberg H
544
- Graham G 247
- Graham G see Archer H E
335
- Graham M L see McHenry
I W 297 301 329
- Grant R see Best C H 540
- Graser E 410
- Cray F L 70
- Gray E L and Cawley J D
70
- Gray E L Hickman K C D
and Brown E F 86
- Gray E L Morgare dge K
and Cawley J D 80
- Gray F I see Baxter J C
61
- Cray L I see Ellis C F 344
401
- Gray I I see Hickman
K C D 384
- Gray P P and Stone I 591
- Gray R I see Robinson H F
264
- Greaves J D 509
- Greaves J D and Schmidt
C L A 56 84 91 418 456
504
- Green D F 4 176 228
- Green D E and Brosteaux J
278 234
- Green D F and Corran H S
175
- Green D E and Dewan J G
173 2 8
- Green D E Dewan J G and
Leloir L F 228
- Green D F Herbert D and
Subrahmanyan V 173
- Green D E Knox W F and
Stumpf P K 171
- Green D E Needham D M
and Dewan J G 228
- Green D E see Corran H S
173 175
- Green D E see Dewan J G
173 174 229
- Green D F see Gordon A H
176
- Green D F see Straub I R
173
- Green D F see Subrahmanyan
V 176
- Green H N 283 28 280
- Greenberg D M see Cohn
W E 424
- Greenberg D M see Tufs
E V 190
- Greenberg L D see Rehner
J F 334
- Greenberg R and Popper H
82
- Greenberg R see Popper H 80
- Greenberg S M see Fraps
G S 53
- Greene D J see Bard I D
410
- Greene J see Bonner J 133
- Greene J A and Swan on
L W 380
- Greene R see Wndaus A
105 110
- Greene R D 199 211
- Greene R D and Back A
102 118 156
- Greenard L 119
- Greenwald C K and Ha de
E 330 333
- Gregory R A 278
- Grelck P M W 557
- Gresl n J see Unna K 270
- Grewe R 100 100 107 109
110
- Grdgeman N T Lees H and
Wlkson H 416
- Grieg J R 428
- Grien W B 410
- Grese A see Warborg O
174 179 235 238
- Griessbach R see Walz F
594
- Griffith W H 44 448
- Griffith W H and M Ford
D J 544 540
- Griffith W H and Wade N J
544
- Griffiths H N H d tch T P
and Rae J 72
- Griffiths J J see Ecker F F
331
- Gribsby H D see Hoffman C
573
- Grjns 9
- Groen J and Schuyt J W
188 192
- Groen J see Schuyt J W 190
- Grönningaeter S 56
- Groll C and Strnmann E
593
- Gross E G see Steenbock H
03
- Gross J see Hess A F 418
419
- Grosser 4 3 4 4
- Grosser and Heymann W 4 3
- Grosman A M see Quack
A J 507
- Grube P 559
- Gruener A G tr fchter M
and Re chsten T 60 71
- Grüssner A and Re chsten T
50 260 261 60
- Grüssner A see Re chsten T
291 296 301 303 306-308
314
- Grundmann C and Takeda Y
49
- Grundmann C see K hn R
39 41 71
- Grundmann W 379
- Grundmann W and Wndaus
A 396
- Gschader B see Wndaus A
350 397
- Guthier E R see Drummond
J C 404
- Günther G see Adler E 174
- Günther G see Euler H v 76
173 222 228 230

- Günther P see John W 441
443 447 450 607
- Cüntzel B see Windaus A
372 376 379 383
- Guerrant N B see Hogan
A G 523
- Guerrant N B see Knight
C A 79
- Guerrant N B see Salmon
W D 109 155
- Guerrant R F see Hogan
A G 541
- Guerry D see Waddell W W
507
- Guggenheim M 2 6
- Guba B C 102 155
- Guba B C and D 4 N 79
- Guba B C and Drummond
J C 102
- Guba B C and Gosh A R
312
- Guba B C and Sen Gupta
P N 230 301 325 3 6
- Guba B C see Ghosh B 239
293 301
- Guba B C s Pal J C 992
301
- Guba B C see S n Gupta
P N 299 301 319
- Gulbert H R 3
- Gulbert H R and Hart
G F 96
- Gulbert H R Howell C E
and Hart G H 9
- Gulbert H R Miller R F
and Hughes F H 96
- Guld B G see Park E A
336
- Gulrmond A Fontaine M
and Raffy A 185
- Guteras A 303
- Guteras A N kamya Z and
Inhoffen H H 375 393
- Gulbransen M C 561
- Gulken T W P Jmer I S
and Boyd W L 432
- Gundel M György P and
Pagel W 477
- Gunnson S 431
- Gunn C Z see Kohman F J
312
- Gurin S see Clarke H T 110
111 113
- Guthrie T and Hygaar A A
319
- Guthrie T s see Hand D B
232
- Gutman M G see He s A I
343 424
- Gutzeit G 79
- György P 181 183 197 199
211 215 260 469-4 1 4 3
474 476 4 8
- György P and Eckardt R F
197 211 344
- György P and Goldblatt H
344 346
- György P Kuhn R and
Lederer E 470 471
- György P Kuhn R and
Wagner Jauregg T 154 155
187 374
- György P Melville D B
Burk D and Vigneaud V
du 470 471
- György P and Loling C F
969 271 471
- György P Poling P C and
Coldblatt H 544
- György P Poling C F and
Subbarow Y 269
- György P and Ro e C S 478
- Cyrgy P Rose C S Hof
mann K Melville D B and
Vigneaud V du 474
- György P Van Klayen T
W Kuhn R and Wagner
Jauregg T 183
- György P see Birch T W
198 199 13 471 472
- György P see Freudenberg E
425
- György P e Gundel M 477
- György P s e Kuhn R 154
156 160
- György P see Vigneaud V du
471
- H
- Haagen-Smith A J see Kogl
F 133 470 475
- Haarhoff H s e Mcheel F
311
- Ha s F 173 174
- Haas F Horecker B L and
Hogne s T R 173
- Haddan H 552
- Häusler C P and Brauchli
F 367 368 413
- Hag C Hecht S and Patek
A J 89
- Hag C ee Hecht S 89
- Haid J see Bndr t 940
- Haiden W 419 596
- Halden W and Tom H 41
- Halden W see Sobotka M
366
- Hall H see Bll C I 384
421
- Hall J M ee Roe J H 336
- Hall L A 605
- Hall day N 213
- Hall day N and E and H M
199 211
- Halp n J G see Knowles
H R 406
- Hamann R W and Steenbock
H 36 368
- Hainano S 59 69 63
- Hamburger T 559
- Hamli B M see Slyker F
4 0
- Hamilton H and Dew r M
M 409
- Hamilton B and Schwartz C
409 490
- Hamilton J D see Rich A R
454
- Hamilton T S and Mitchell
H H 183
- Hammarsten O 505
- Hammett F S and Walp L
549
- Hanan 4 7
- Hand D B 189
- Hand D B Guthrie E S and
Sharp P F 299
- Handley W R C see Evans
W C 964
- Hanford 7 M see Supplee
G C 18
- Hanke H 333
- Hansen A F 330
- Hanse A F and Burr G O
539
- Hansen A F Wilson W R and
Williams H H 539
- Happold F C see Evans
W C 264
- Harde E 331
- Harde F see Greenwald C K
330 333
- Harden A and Zil a S S
990
- Harrington C R and Mogg
ridge R C G 119
- Harmapp G O 410 431
- Harjer H A and Denel H J
148
- Harper S H see Batty J W
90
- Harrel C G and Lindert A
W 556
- Harrison R T see Sure B
339
- Harris C J and King C G
399
- Harris C J s e Stotz L 3 7
- Harris B R see Cahn F J
557
- Harris I I 554 573
- Harris L J 0 59
- Harris I J and Innes J R
M 430
- Harris I J and Leong P C
147
- Harris L J Iong I C and
Ungley C C 147
- Harris L J Mills J I and
Innes J R M 323
- Harris L J and Moore T W
380 4 6
- Harris I J Pa more R and
Pagel W 331
- Harris L J and Ray S N
393 375 330
- Harris L J and Raymond
W D 940 244 948
- Harris L J s Abbasy M A
331
- Harris I J see H nerji G C
149

- Harris L J see Brech T W
 113 131 147 213 318
 Harris L J see Wang Y L
 147
 Harris P L see Baxter J G
 60
 Harris R S Bunker J W M
 and Mosher L M 368
 Harris R S see Bunker
 J W M 369
 Harris S A 584
 Harris S A and Folkers K
 204
 Harris S A Stiller F T and
 Folkers K 204
 Harris S A see Keresztesy
 J C 198
 Harris S A see Stiller F T
 255 267 261 284 271
 Harrison D C 228
 Harrison D C see Haw
 thorne J R 313
 Harrow B Power F W and
 Sherwin C P 285
 Hart E B see Arnold A 541
 Hart E B see Hegsted D M
 214 216 474 477 522 541
 542 545
 Hart E B see Keenan J A
 521
 Hart E B see Kline O L
 521 522
 Hart E B see Knowles H R
 406
 Hart E B see Kohler G O
 526
 Hart F B see Oleson J J
 214 234 269 477
 Hart E B see Rupel I W
 432
 Hart E B see Steenbock H
 385
 Hart E B see Wegner M I
 150 194 216 250 271 478
 Hart G H see Guilbert H R
 95 97
 Hartenstein H J 410
 Hartley P 537
 Hartman J I see Gersten
 berger H J 425
 Hartmann M and Panizzon
 L 585
 Hartmann M and Seiberth
 M 585
 Hartree E F see Keilin D
 327
 Harvey E N 404
 Harvey S C see Taffel M
 333
 Haslewood G A D 360 361
 407 408
 Haslewood G A D and Drum
 mond J C 344 387 388
 Hassan A and Drummond
 J C 156
 Hasse K see Euler H v 173
 Hasselt W van see Kogel F
 276 470
 Hastings A B 424
 Hastings A B Murus J and
 Bessey O A 190
 Hastings A B see Barron
 E S C 160
 Hastings A B see Conant
 J B 542
 Hastings A B see Hutchens
 J O 250
 Hastings A B see Jandorf
 B J 234
 Hastings A B see Solomon
 A K 542
 Hathaway M L and Lobb
 D E 362
 Hatv F B see Armentanó I
 517
 Hauck H M see Belser
 W B 335
 Hausen D A 564
 Hausen K H see Olefabrik
 Aarhus 570
 Hausen S von 323
 Hausmann F see Butenandt
 A 407 408
 Havemann R see Wolff K
 603
 Hawkes C D see Richter
 C P 213
 Hawkins W B and Brinkhous
 K M 509
 Hawley E E Frazer J P
 Button L L and Stevens
 D L 336
 Hawley F E and Stephens
 D J 325 326
 Hawley E E see Stephens
 D J 326
 Haworth W N 291 307
 Haworth W N Hirst E L
 Jones J K N and Smith
 F 584
 Haworth W N see Ault
 R G 291 308
 Haworth W N see Baird
 D K 291 308
 Haworth W N see Szent
 Györgi A 289
 Hawthorne J R and Harrison
 D C 313
 Hays I M see Salmon W D
 102 155
 Hazey V see Smith E L 79
 Hazura K see Wedel H
 224
 Heath W P 554
 Hecht S 89
 Hecht S Chase A M and
 Shaler S 89
 Hecht S Chase A M
 Shaler S and Haug C
 89
 Hecht S and Mandelbaum J
 89 93
 Hecht S and Shaler S 93
 Hecht S see Haug C 89
 Hecker J C see Hickman
 K C D 567 568
 Heffele G 553
 Helts P see Bosshard R 576
 Hegarty C P see Rahn O
 324
 Heggie R see Milas N A
 362
 Hegsted D M Briggs G M
 Elvehjem C A and Hart
 E B 522 541
 Hegsted D M Mills R C
 Briggs G M Elvehjem
 C A and Hart E B 477
 Hegsted D M Mills R C
 Elvehjem C A and Hart
 E B 545
 Hegsted D M Ole on J J
 Elvehjem C A and Hart
 F B 214 210 542
 Hegsted D M Ole on J J
 Mills R C Elvehjem C A
 and Hart F B 474 477
 Heiduschka A and Lindner
 H 385 387
 Heilbron I M 601
 Heilbron I M and co workers
 65 80
 Heilbron I M and Batty
 J W 572
 Heilbron I M Gillam A E
 and Morton R A 72 79
 Heilbron I M Heslop R N
 Morton R A Webster F
 T Rea J L and Drum
 mond J C 60 61 63
 Heilbron I M Jones R N
 Samant K M and Spring
 F S 396
 Heilbron I M and Jones
 W E 65
 Heilbron I M Kamm F D
 and Morton R A 343
 Heilbron I M and Lythgoe
 B 42
 Heilbron I M Morrison
 A L and Simpson J C E
 354
 Heilbron I M Morton R A
 and Webster F T 69
 Heilbron I M and Spring
 F S 367 368 376 413
 Heilbron I M Spring F S
 and Stewart P A 376 399
 Heilbron I M see Barr T
 360
 Heilbron I M see Batty J W
 70
 Heilbron I M see Castle
 D C 63
 Heilbron I M see Edsbury
 J R 62
 Heilbron I M see Gillam
 A E 59 68 70 79
 Heilbron I M see Morton
 R A 57
 Heiman M 180
 Heiman V see Norris L C
 185
 Hennemann F 60

- Heinemann M 317
 Heinemann M see Eekelen
 M van 495 326
 Heintz E 295
 Hentan H 602
 Heitman H see Jukes T H
 131
 Heiwinkel H see Euler H v
 2 30 233 235 236 240
 244
 Helfenstein H see Karrer P
 31
 Helfenich B 311 589
 Helfer ch B and Peters O
 310 588
 Helholt K 565
 Hellerud R see Langfeldt F
 562 563
 Hellman L M see Shettles
 L B 505
 Hellström H see Adler I 232
 Hellström H see Fuler H v
 38 75 173 174 2 8 230 2 6
 3 8
 Hellström H see Myrbäck K
 230 231 236
 Helman F D see Hess A F
 343
 Helmer A C and Jansen C
 405
 Himer O M see Fouts P J
 214 220 247 254
 Hemd K 371 60
 Hemingway A see Wood H
 G 139 141
 Hendrick F G see Smith
 M J 156
 Hendricks J B see Morgan
 A F 430
 Hennessy D J see Cecedo
 L R 118
 Henriksen P K 599
 Henry K M see Campion J
 E 403 406
 Heitschel H and Schadel L
 405
 Heitschel H and Zöllner E
 426
 Hill M s Ellinger A
 285
 Herding L see Kuhn R
 497
 Herbert D see Green D E
 123
 Herbert R W Hirst E L
 Percival E G V Reynolds
 R J W and Smith F 297
 299
 Herbert R W Percival E G
 V Reynolds R J W Smith
 F and Hirst E L 991
 Herbert R W see Ault R G
 991 308
 Herbert R W see Bard D K
 291 308
 Herbert R W see Hirst E L
 298
 Herrmann 426
 Hershey J M see Best C H
 543 544
 Herzberg H 245
 Herzog J see Weidel H 224
 Heslop R N see Heilbron
 I M 60 61 63
 Hess A F 343 422
 Hess A F and Benjamin
 H R 424
 Hess A F and Gutman M G
 343 424
 Hess A F and Lewis J M
 410
 Hess A F Lewis J M and
 Rivkin H 410
 Hess A F Light R F Frey
 C N and Gross J 418 419
 Hes A F and Unger L J
 292 293 343
 Hess A F and Weinstock M
 343 405 419
 Hess A F Weinstock M and
 Gross J 418 419
 Hess A F Weinstock M and
 Helman F D 343
 Hess A F see Benjamin H R
 424
 Hess A F see Windaus A 343
 Hester J B 313
 Heus G F Wilgus H s and
 Norris L C 191 194
 Heuser G F see Bauernfeind
 J C 2 3
 Heuser G F see Davis H J
 191 194
 Heuser G F see Norris L C
 185
 Heuser G F see Ringrose
 A T 254 269 478
 Heuser G F see Schumacher
 A E 191
 Heuser G F see Wilgus H S
 523
 Heyerdahl P M 561 564 566
 Heymann W 418
 Heymann W see Grosier 423
 Heyns K 131
 Heyns K see Dimer O 307
 589 590
 Hickman s and co workers 67
 Hickman J O and Hickman
 N V 601
 Hickman K C D 59-61 80
 388 566-569
 Hickman K C D and Gray
 E L 384
 Hickman K C D and Hecker
 J C 567 568
 Hickman K C D and Tischler
 A O 567 568
 Hickman K C D see Baxter
 J G 60
 Hickman K C D see Ellis
 C E 344 401
 Hickman K C D see Gray
 E L 86
 Hickman N V see Hickman
 J O 601
 Higgins C C 92
 Hightower P see Spies T D
 214 215 249 270 464 523
 Hilditch T F see Griffiths
 H N 72
 Hilger 572
 Hill N G see Abbasy M A
 3 0
 Hills G M 128 140 147
 Hilston N W 432
 Himsforth H P 544
 Hinchich H E Goldfarb W
 and Cowgill C R 145
 Hind H G see El Sadr M M
 264
 Hines H M see Knowlton
 G C 462
 Hines H M see Wood E L
 462
 Hinglas H see Meunier P
 503 509
 Hirota O 427
 Hirsch P see Tillmans J 291
 320
 Hirschberger C see Schiff E
 575
 Hirst E L 298
 Hirst E L and co-workers 291
 Hirst E L Percival E G V
 Herbert R W Reynolds
 R J W and Smith F 298
 Hirst E L Percival E G V
 and Smith F 298 299
 Hirst E L see Ault R G 291
 301
 Hirst F L see Bard D K
 291 308
 Hirst E L see Cox E G 294
 997
 Hirst E L see Haworth W N
 588
 Hirst E I see Herbert R W
 291 297 299
 Hitchings G H see Subbarow
 Y 256 266 269 270
 Hoagland R 101
 Hoar S B see Stephens H C
 599
 Hodson A Z and Norman L
 C 182
 Hodson A Z see Bauernfeind
 J C 253
 Hoefelmayer K 600
 Hoefler 8
 Höggberg B see Euler H v
 145 221 2 2 230 233 235
 240 241 214 450 529
 Hoehn H H see Evans H M
 449 451
 Höjer J A 3 3
 Höllering H F see Mitrack
 J 147
 Hölcher A 560
 Hofer J W see Stern K G
 125
 Hofer M 579
 Hofer M and Reichstein T
 264

- Harris I J see Birch T W
 113 131 147 213 318
 Harris L J see Wang Y L
 147
 Harris P L see Baxter J G
 60
 Harris R S Bunker J W M
 and Mosher L M 368
 Harris R S see Bunker
 J W M 369
 Harris S A 584
 Harris S A and Folkers K
 204
 Harris S A Stiller F T and
 Folkers K 204
 Harris S A see Feresztely
 J C 198
 Harris S A see Stiller F T
 255 267 261 264 271
 Harrison D C 228
 Harrison D C see Haw
 thorne J R 313
 Harrow B Power F W and
 Sherwin C P 285
 Hart E B see Arnold A 541
 Hart E B see Hegsted D M
 214 216 474 477 522 541
 542 545
 Hart E B see Keenan J A
 521
 Hart E B see Kline O L
 521 522
 Hart E B see Knowles H R
 406
 Hart E B see Kohler G O
 526
 Hart E B see Oleon J J
 214 254 269 477
 Hart E B see Rupel I W
 432
 Hart E B see Steenbock H
 385
 Hart E B see Wegner M I
 150 194 216 250 271 478
 Hart G H see Guilbert H R
 95 96
 Hartenstein H J 410
 Hartley P 532
 Hartman J I see Gersten
 berger H J 425
 Hartmann M and Panizzon
 L 585
 Hartmann M and Seiberth
 M 585
 Hartree E F see Keilin D
 327
 Harvey E N 404
 Harvey S C see Taffel M
 333
 Haslewood G A D 360 361
 407 408
 Haslewood G A D and Drum
 mond J C 344 387 388
 Hassan A and Drummond
 J C 156
 Hasse K see Euler H v 173
 Hasselt W van see Kogi F
 276 470
 Hastings A B 424
 Hastings A B Muus J and
 Bessey O A 190
 Hastings A B see Barron
 E S G 160
 Hastings A B see Conant
 J B 542
 Hastings A B see Hutchens
 J O 250
 Hastings A B see Jandorf
 B J 234
 Hastings A B see Solomon
 A K 342
 Hathaway M L and Lobb
 D E 362
 Hatz F B see Armentanó I
 517
 Hauck H M see Belser
 W B 335
 Hausen D A 564
 Hausen K H see Olef brik
 Aarhus 570
 Hausen S von 323
 Hausmann E see Butenandt
 A 407 408
 Havemann R see Wolff K
 603
 Hawkes C D see Richter
 C P 213
 Hawkins W B and Brinkhous
 K M 509
 Hawley E E Frazer J P
 Button L L and Stevens
 D L 336
 Hawley E E and Stephens
 D J 325 326
 Hawley E E see Stephens
 D J 326
 Haworth W N 291 307
 Haworth W N Hrst F L
 Jones J K N and Smith
 F 588
 Haworth W N see Ault
 R G 291 308
 Haworth W N see Baird
 D K 291 308
 Haworth W N see Szent
 Györgi A 289
 Hawthorne J R and Harrison
 D C 313
 Hays I M see Salmon W D
 102 155
 Hazley V see Smith E L 79
 Hazura K see Wedel H
 224
 Heath W P 554
 Hecht S 89
 Hecht S Chase A M and
 Shaler S 89
 Hecht S Chase A M
 Shaler S and Haig C
 89
 Hecht S and Mandelbaum J
 89 91
 Hecht S and Shaler S 93
 Hecht S see Haig C 89
 Hecker J C see Hickman
 K C D 567 568
 Heffele G 553
 Hefti P see Bosshard R 574
 Hegarty C P see Rahn O
 324
 Heggie R see Milas N A
 362
 Hegsted D M Briggs G M
 Elvehjem C A and Hart
 E B 522 541
 Hegsted D M Mills R C
 Briggs G M Elvehjem
 C A and Hart E B 477
 Hegsted D M Mills R C
 Elvehjem C A and Hart
 E B 545
 Hegsted D M Oleon J J
 Elvehjem C A and Hart
 F B 214 216 549
 Hegsted D M Oleon J J
 Mills R C Elvehjem C A
 and Hart F B 474 477
 Heiduschka A and Lindner
 H 360 367
 Heilbron I M 603
 Heilbron I M and co workers
 65 80
 Heilbron I M and Batty
 J W 572
 Heilbron I M Gillam A F
 and Morton R A 72 79
 Heilbron I M Heslop R N
 Morton R A Webster F
 T Rea J L and Drum
 mond J C 60 61 63
 Heilbron I M Jones R N
 Samant K M and Spring
 F S 396
 Heilbron I M and Jones
 W E 65
 Heilbron I M Kamm F D
 and Morton R A 343
 Heilbron I M and Lythgoe
 B 42
 Heilbron I M Morrison
 A L and Simpson J C E
 354
 Heilbron I M Morton R A
 and Webster F T 69
 Heilbron I M and Spring
 F S 367 368 376 413
 Heilbron I M Spring F S
 and Stewart P A 376 399
 Heilbron I M see Barr T
 360
 Heilbron I M see Batty J W
 70
 Heilbron I M see Castle
 D C 63
 Heilbron I M see Edisbury
 J R 62
 Heilbron I M see Gillm
 A E 59 68 70 79
 Heilbron I M see Morton
 R A 59
 Heiman M 186
 Hemman V see Norris L C
 185
 Heintemann F 607

- Heinemann M 317
 Heinemann M see Bekelen
 M van 325 326
 Heintz E 993
 Heintz H 60
 Heintz H see Jukes T H
 131
 Heinkel H see Euler H v
 222 230 233 235 236 240
 244
 Helfenstein H see Karrer F
 51
 Helfrich B 311 589
 Helfrich B and Peters O
 310 589
 Helholt K 565
 Hellerud R see Langfeldt E
 56 565
 Hellman L M see Shettles
 L B 505
 Hellström H see Adler E 232
 Hellström H see Euler H v
 38 75 173 174 228 230 236
 329
 Hellström H see Myrbäck K
 230 231 236
 Helman F D see He s A F
 343
 Helmer A C and Jans n C
 405
 Helmer O M see Fout P J
 214 290 247 54
 Hembl K 371 607
 Hengswy A s e Wood H
 C 139 141
 Hendrick F G s e Smith
 M I 156
 Hendrick J B see Morgan
 A F 430
 Hennsy D J see Cerceto
 L R 118
 Henriks n P K 592
 Henry K M see Campion J
 E 403 406
 Hentschel H and Schnd l L
 405
 Hentschel H and Zöller E
 426
 Heazel M ee Elling r A
 285
 Heppug L e Kühn R
 497
 Herbert D see Green D E
 123
 Herbert R W Hirst E L
 Percival E G V Reynolds
 R J W and Smith F 297
 299
 Herbert R W Percival E G
 V Reynolds R J W Smith
 F and Hirst E L 291
 Herbert R W s e Ault R G
 291 308
 Herber R W s e Baird D K
 291 308
 Herbert R W s e Hirst E L
 298
 Herrmann 426
 Hershey J M se Best C H
 543 544
 Herrenberg H 745
 Herzog J see Weidel H 224
 Heslop R N see Halbron
 I M 60 81 83
 Hess A F 343 422
 Hess A F and Benjamin
 H R 424
 Hess A F and Gutman M G
 343 424
 Hess A F and Lewis J M
 410
 Hess A F Lewis J M and
 Rivkin H 410
 Hess A F Light R F Frey
 C N and Gross J 418 419
 He s A F and Unger L J
 292 295 343
 Hess A F and Weinstock M
 343 405 419
 He s A F Weinstock M and
 Gross J 418 419
 Hess A F Weinstock M and
 Heiman F D 343
 Hess A F see Benjamin H R
 424
 Hess A F see Windaus A 343
 Hirst J B 313
 Huser G F Wilgus H S and
 Norris L C 191 194
 Huser G F see Bauernfeind
 J C 53
 Huser G F see Davis H J
 191 194
 Huser G F see Norris L C
 185
 Heu r G F se Ringrose
 A T 254 69 478
 Heuser G F see Schumacher
 A E 191
 Heuser G F see Wilgus H S
 523
 Heyerdahl P M 561 564 566
 Heymann W 418
 Heymann W ee G osser 423
 Heyns K 131
 Hyns K se Dalmer O 307
 589 590
 Hickman and owo kers 67
 Hickman J O and Hickman
 N V 601
 Hickman K C D 59-61 80
 388 566-569
 Hickman K C D and Gray
 E L 384
 Hickman K C D and Hecker
 J C 567 568
 Hickman K C D and Tischer
 A O 567 568
 Hickman K C D see Baxter
 J G 80
 Hickman K C D ee Blis
 C E 344 401
 Hickman K C D s e Gray
 E L 86
 Hickman N V s e Hickman
 J O 601
 Higgins C C 92
 Hightower P see Spies T D
 114 215 249 270 464 523
 Hilditch T P see Griffiths
 H N 72
 Hilger 572
 Hill N G se Abbasy M A
 330
 Hills G M 128 140 147
 Hilston N W 437
 Himsforth H P 544
 Hinch H E Goldfarb W
 and Cowgill G R 145
 Hind H G see El Sadr M M
 264
 Hines H M see Knowlton
 G C 462
 Hines H M see Wood E L
 465
 Hinglais H see Meunier P
 503 509
 Hirota O 427
 Hirsch P see Tillmans J 791
 320
 Hirschberger C s e Schiff E
 5 5
 Hirst E L 298
 Hirst E L and co-workers 291
 Hirst E L Percival E G V
 Herbert R W Reynolds
 R J W and Smith F 298
 Hirst E L Percival E G V
 and Smith F 298 99
 Hirst E L see Ault R G 291
 301
 Hirst F L see Baird D K
 291 308
 Hirst E L see Cox E G 294
 297
 Hirst E L see Haworth W N
 588
 Hirst E L see Herbert R W
 291 297 299
 Hitchings G H see Subbarow
 Y 56 266 769 270
 Hoagland R 101
 Hoar S B see Stephens H C
 599
 Hodson A Z and Norris L
 C 182
 Hodson A Z s e Bauernfeind
 J C 253
 Hoeflmayr K 600
 Hoefler 8
 Höglberg B se Euler H v
 145 211 222 230 233 235
 240 241 244 250 5 2
 Hoehn H H see Evans H M
 449 451
 Höjer J A 323
 Höllering H F see M rrack
 J 147
 Hölscher A 560
 Hel r J W see Stern K C
 175
 Hoff r M 579
 Hoffer M and Reichst n T
 264

- Hoffert D see Smedley Mac
 Lean I 365
 Hoffman C 556
 Hoffman C Grigsby H D
 and Cregor N M 554 573
 Hoffman F and Marquardt
 P 591
 Hoffman G R see Smith
 H P 507 508
 Hoffman G R see Ziffern
 S E 508
 Hofman M M see Schour
 L 93
 Hoffman O see Karrer P
 449
 Hoffman R M and Daniels F
 371
 Hoffman U see Muller J 590
 Hofman E see Elderkin J A
 599
 Hofmann K Melville D B
 and Vigneaud V du 472
 Hofmann A see György P
 474
 Hofmann A see Melville
 D B 472
 Hofmann K see Vigneaud V
 du 470-472
 Hofmeister F 130 543
 Hofstra see Nickerk J van
 419
 Hogan A G 197
 Hogan A G Guerrant N B
 and Kempster H L 523
 Hogan A G and Parrott
 E M 523
 Hogan A G Powell E L and
 Guerrant R E 541
 Hogan A G Richardson L R
 and Patrik H 593
 Hogan A G Richardson L R
 Patrik H and Kempster
 H L 593
 Hogan A G and Shrewsbury
 C L 523
 Hogan A G see Richardson
 L R 197 286
 Hogan A G see Robbins
 W J 196 133
 Hoglan F A see Bartow E
 277
 Hogness T R Sidwell A E
 and Zscheile F P 350
 Hogness T R see Haas E 173
 Holaday D see Williams R J
 753 254 257
 Holaday H A and Black A
 562
 Holcomb W F see Binkley
 S B 482 487 493
 Holcomb W F see Mac
 Corquodale D W 493
 Holm E 89
 Holm E see Frederica L S
 89
 Holmes C E and Cravens
 W W 463
 Holmes H N 563 570
 Holmes H N Amberg E J
 and Campbell K 330 334
 Holmes H N and Corbet
 R E 37 60-62 72 75 81
 Holmes H N and Leicester
 H M 569
 Holmes A D Tripp F
 Woelffer E A and Satter
 field G H 376
 Holst A 289 290
 Holst A and Frölich T 9
 790
 Holt R L see Kinter J H
 432
 Holtz F 380 425
 Holten H see Sah P P T
 491
 Honeywell H E see Bechdel
 S I 101 150
 Honeywell E M see Bills
 C E 348 370 371 380
 Honigmann H 358
 Hood J S and Ravitch I
 410
 Hoogewerf S and Dorp W
 A van 224 267
 Hooper C W 405
 Hoover A A see Drummond
 J C 438
 Hoover S R see Allison F E
 469 474 475
 Hopkins F G 9 11 12 37
 297 295 342
 Hopkins F G and Morgan
 F J 3-7
 Hopkins F G see Crook E
 M 327
 Hoppert C A see Bechtel
 H E 403 406
 Horecker B L see Haas E
 173
 Horwitt M K see Dorfman
 A 249
 Hottle G A Lampen J O
 and Pappesheimer A M
 475
 Hou H C and Tso E 406
 Houston J and Kon S K
 101 130 147
 Houston J Kon S K and
 Thompson S Y 101 147
 Howe P R see Wohlbach
 S B 92 93 330
 Howell C E see Guilbert
 H R 95
 Howland J and Kramer B
 420
 Hoyle J C 380
 Hoyle J C and Buckland H
 380
 Hoyle J C see Dixon W E
 380
 Hromatka O 106 112 577
 579 580 608
 Hu C A see Frazer C N
 92
 Hubbard L H see Spies T D
 214 215 249 270 464 523
 Huber C 273 224
 Hubers P J see Jansen
 B C P 10-
 Hubert L see Drumel G 471
 Hübner H and Verzar F 179
 Huebschmann H see Kuhn
 R 142
 Hünecke H 222
 Huff J W see Perlzweig
 W A 244
 Huff N E see Spies T D
 191
 Hughes E H 191
 Hughes B H see Guilbert
 H R 96
 Hughes J S see Scott H M
 410
 Kuhn O see Widenbauer F
 178
 Huldshinsky K 342 425
 Hult H 507
 Hume E M 81 343 4-5
 Hume E M and Chick H
 61 81
 Hume E M Lucas N S and
 Smith H H 417
 Hume E M Nunn L C A
 Smedley MacLean I and
 Smith H H 536 537 539
 Hume E M see Smedley Mac
 Lean I 538
 Hummel F C see Hunscher
 H A 430
 Hummel R see Schönheimer
 R 366
 Hummoller F L see Austin
 W C 170
 Hunscher H A Hummel
 F C and Macy I G 430
 Hunt D J see Sebrell W H
 242
 Huntsman M E see Best
 C H 543 544
 Hurlock E see Porter C M
 557
 Hurxthal L M and Claiborne
 T S 380
 Huston R C and Lightbody
 H D 563
 Huszak I 517
 Hutchens J O Jandorf B J
 and Hastings A B 250
 Hutchings B L and Woolley
 D W 212
 I
 Ikegaki I 95
 Imai T see Makino K 107
 113
 Imboden M see Bills C E
 344 384 411
 Ingalls T H see Lanman
 T H 333
 Inhoffen H H 356 357 375
 398

- Inhoffen H H see Guter s A
 370 393
 Inhoffen H H see Windaus
 A 354
 Ino s J R M see Harris L J
 323 430
 Inukai B see Nakahara W
 524 581
 Ipatiev W 304
 Ireland J s e l o v e r n J A 70
 Irv n W B see Sm th L I
 453
 Ir ing J T and Richard M
 B 07 93 96
 Isa cs B see Elliott M C
 003 504
 Isbell H S s e Ellis N R
 038
 Isbell H s e Frazer H F 188
 197
 Isbell H see Sebrell W H
 193
 Isbell H see Sydenstricker
 V P 477
 Iscovesco H 555
 I co esco H and Adams A B
 564
 Ishii T s e Karrer P 239
 Isler O 448 449 609
 Isler O see Demole V 449
 Itib A and Miti K 198 199
 01 20 006 07
 Itt r S Orient E R and Mc
 Collum E V 156
 Iversen P and Ienstrup I
 420
 Ivey G see Todhunter E N
 516
 Ivy A C see Elliott M C
 503 504
 Iwatake D see Fujita A 30
 Izume S Sato M and Seto I
 575

 J
 J kson D see Park E A
 336
 Jackson H C see Wel kel A
 G 403
 J cob A Steiger M and Todd
 A R 446
 Jacob A Sutcliffe F A and
 Todd A R 449
 Jacob A see Bergel F 440
 Jacob A see Todd A R 111
 120
 Jacobi H P Baumann C A
 and Meek W J 547
 Jacobi M see Zuckerman I C
 507
 J eger C 600
 Jaeg r W see Karrer P 437
 450
 Jafté W see Karrer P 39
 Jafté E see Tortelli M 413
 Jafté H L see Bodinsky A
 424
 Jahn A R 559
 Jandori B J 230
 Jandori B J Klempere r P
 W and Hastings A B 234
 Jandori B J see Hutchens
 J O 250
 Janeway C A 486
 Janota M and Dack G M
 5 4
 Jansen B C P 99 102 128
 Jansen B C P see Dols M J
 L 423
 Jans n B C P and Donath
 W P 100 101
 Jan en B C P Kinnersley
 H W Peter R A and
 Reader V 102
 Jansen B C P Wibaut J B
 Hube s P J and Wiard P
 W 101
 Jan en B C P see West n
 b nk H G F 130 147
 Jansen C s Helmer A C
 405
 Jans n E see Lunde G 253
 Jansen J see Nicolaysen R
 422
 Jeans P C 410
 Jean P C Bl nchard F and
 Zentmire Z 91
 Jeans P C and Stearn G
 430
 Je us P C and Zentmire Z
 81 92
 Je n s P C see Stearns C 470
 Jeffreys C E P see Borsook
 H 295 298 327
 Jeffreys G A 573
 J fremow W W 213
 Jeghers H 97
 Jena H and Jena J 559
 Jena J see Jena H 559
 Jendras k A 603
 Jendras k A and Keményfi
 A G 367
 J ney A v and Torø E 330
 J nsen H B see Willstaedt
 H 72
 Jens n K A see Karrer P
 450
 Jensen O G s Supplee
 G C 183
 Jense R M see Flot M M
 412
 Jersey V 569
 Jers ld T 518
 Jetter W W and Bumbalo
 T S 30 333
 Jewell W J see C r r F H 61
 568
 Je ler A and Niederberg r
 W 336
 John W 436 441 443 452
 John W nd D lmer O 609
 John W Dietz l E and Emst
 W 449 457
 John W Dietzel E and
 Cunth r P 441 443
 John W and G nther P 447
 607
 John W Gunther P and
 Schme l M 447 450
 John W and Schmeil M
 447
 Johnson B C Elvehjem C A
 Peterson W H and Fagen
 H J 5 f
 Johnson F M 561
 Johnson G D 507
 John on H s e Dimroth A
 402
 John on J Y 611
 John on I v see Eckardt
 R J 189 191
 Johnson M L 600
 Johnson R E see Thompson
 R H S 136 149
 Johnson R H see Carver J S
 420
 Johnson W A see Krebs H
 A 137
 Johnston E S 84
 Jo ner R R s e Schmelk s
 F C 177
 Jolliffe N 146
 Jolliffe N Bowman F M
 Rosenblum L A and Fein
 H D 246
 Jolliffe N see Rosenblum L A
 248
 Jones 57
 Jones J H and Cohn B N E
 4 5
 Jones J K N see Haworth
 W N 588
 Jones L W and Major R T
 611
 Jones R L 99 219 289 341
 430
 Jon s R N s Pieser L F
 496
 Jones R N s e Heilb on I M
 396
 Jones W A and Cure B 146
 Jones W E see Batty J W
 70
 Jones W E see G lham A E
 59 70
 Jones W E see He lbron I M
 65
 Jones W S see Christiansen
 W G 56
 Jones W S s e Nitardy F W
 563
 Jorgensen P S 941
 Josephson E M and K lewan
 G 947
 Jo serand A see Arlong F
 593
 Jowett M 147
 Joyce F T see Tidrick R T
 502
 Judowitz M and Verzar F
 188
 Juhász Schäffer A 406 458
 463

- Jukes T H 184 214 255 266
 267 269 270 523 545
 Jukes T H and Heitman H
 131
 Jukes T H and Lepkovsky S
 253 266
 Jukes T H and Sanford T D
 384 410
 Jukes T H see Babcock S H
 260
 Jukes T H see Daft I S 269
 271
 Jukes T H see Fouts P J
 214 220 254
 Jukes T H see Lepkovsky S
 188 191 197 253 254 257
 269 270 477
 Jukes T H see Spies T D
 268
 Julius H W see Eckelen M
 van 79
 Jung A see Schopfer W H
 131 134
 Jung F 608
 Jung F see Möller E F 209
 211
 Jung F see Werder F v 449
 450
 Jungblut C W 330
 Jungblut C W and Zwemer
 R I 331
 Jungberr E see Pappenheimer
 A M 463
 Jussatz H J 426
- K**
- Kahler H and Davis E P
 182
 Kahnt F W see Karrer P
 239
 Kaiser E W see Smith I I
 447
 Kallman O see Bleyer B
 154
 Kaltschmitt H see Kuhn R
 155 183 187-189 192
 Kamen M D see Ruben S
 137
 Kamm E D see Heitbron
 I M 343
 Kamm O see Ewing D T
 485 486 503
 Kark R and Lozner E L 507
 509
 Karlson S see Euler H v
 234
 Karimullah see Todd A R
 111
 Karrer P 37 57 62 69 72
 501 581 582 583 607 609
 Karrer P and co workers 41
 46
 Karrer P Becker B Benz F
 Frei P and Schöpp K 160
 Karrer P and Benz F 238
 Karrer P and Bussmann G
 449
 Karrer P and Epprecht A
 496
 Karrer P Escher R Fritzsche
 H Keller H Ringier
 B H and Salomon H 442
 444 450 453
 Karrer P Fuler H v and
 Hellström H 328
 Karrer P Euler H v Malm
 berg M and Schöpp K
 181
 Karrer P Fuler H v Malm
 berg M Schöpp K and
 Benz I 181
 Karrer P Frei P and Meer
 wein H 177 179
 Karrer P Frei P Ringier
 B H and Bendas H 179
 Karrer P and Fritzsche H
 107 435 445 449
 Karrer P Fritzsche H Ringier
 B H and Salomon H 437
 445
 Karrer P and Geiger A 441
 457 481 484 485
 Karrer P Geiger A Legler
 R Rüegger A and Salomon
 H 483
 Karrer P Helfenstein H
 Wehrliand H and Wettstein
 A 51
 Karrer P and Hoffmann O
 449
 Karrer P Jaeger W and
 Keller H 437 456
 Karrer P and Jensen K A
 450
 Karrer P Kahnt F W
 Epstein R Jaffé W and
 Ishii T 239
 Karrer P and Keller H
 221 241 247 453
 Karrer P Koenig H Ringier
 B H and Salomon H 442
 445 449
 Karrer P and Kubli U
 128
 Karrer P Legler R G and
 Schwab G 450
 Karrer P Loszt L and
 Verzar P 477
 Karrer P and Meerwein H
 165
 Karrer P and Morf R 61
 63
 Karrer P Morf R and
 Schöpp K 54 60
 Karrer P and Ostwald R
 162
 Karrer P and Quibell T H
 181
 Karrer P Ringier B H
 Büchi J Fritzsche H and
 Solmsen U V 233 238
 Karrer P and Rüegger A
 66
 Karrer P Rüegger A and
 Geiger A 69 70 76
 Karrer P and Rydbom M
 76
 Karrer P and Salomon H
 46
 Karrer P Salomon H and
 Fritzsche H 449 457
 Karrer P Salomon H Morf
 R and Schöpp K 296
 298
 Karrer P Salomon H Morf
 R and Walker O 65
 Karrer P Salomon H and
 Schöpp K 156
 Karrer P Salomon H Schöpp
 K Benz F and Becker B
 167 181
 Karrer P Salomon H Schöpp
 K and Morf R 296 298
 Karrer P Salomon H Schöpp
 K and Schlitter E 164
 Karrer P Salomon H Schöpp
 K Schlitter F and Fritzsche
 H 163 181
 Karrer P Salomon H Schöpp
 K Schlitter E and Wagner
 Jauregg T 160
 Karrer P and Schlients W
 38 41
 Karrer P Schlitter E Pfahler
 K and Benz F 164
 Karrer P and Schöpp K 163
 72 80
 Karrer P Schöpp K and
 Benz F 167
 Karrer P Schöpp K Benz
 F and Pfahler K 167
 Karrer P and Strong F M
 167
 Karrer P Schwarzenbach G
 Benz F and Solmsen U V
 238
 Karrer P Schwarzenbach G
 and Schöpp K 296-298
 Karrer P Schwarzenbach G
 and Utringer G E 238
 Karrer P and Solmsen U V
 76
 Karrer P and Walker O 44
 Karrer P and Warburg O
 238
 Karrer P and Yap K S
 450
 Karrer P see Dam H 482
 496 497 503 505
 Karrer P see Demole V 441
 Karrer P see Euler H v 53
 54 70 72 75 76 79 167 181
 185 232 331
 Karrer P see Kuhn R 154
 Karrer P see Zechmeister L
 37
 Karstens A see Diels O 3
 Kusahara M and Kawamura
 R 337
 Kaselitz O see Kuhlning O
 163
 Kaslow M see Ralli F I
 325

- Kass R J and Roe J H
 335
 Kaszuba F J see Cerec do
 L R 118
 Kato K 508
 Katzman M B 556
 Katznelson M M see Knun
 ya ts I I 586
 Kaufmann H B 566
 Kaufmann O see Wind us A
 363
 Kavanaugh F see Robbins
 W J 127
 Kawai K 56 565 570 571
 Kawakami K 50 63
 Kawamura R see Kasah ra
 M 337
 Kay H D 4 3 429
 Kay H D see Folley S J
 4.3
 Kedd A 560
 Keen n G L see Nelson E
 L 276
 K. na J A Kline O L
 Elvehjem C A and Hart
 E B 571
 Keeton R W see Spilberg
 M A 544
 Keil n D and Hartre E F
 327
 Keil n D and Mann T 327
 Kem tsu b Yokata K. and
 S toda I 2 J
 Kekwick R A and Pederson
 K. O 172
 K. ller H see Karrer P 221
 241 247 437 442 444 450
 453 456
 Keller R see Todd A R 111
 Kelley E see Lease J G 477
 478
 Kelly O R and B y W E
 508
 Kelly O R see Br y W E
 508
 K. lly T D 600
 Keményfi A G s Jendra k
 A 367
 K. mm r A R see Fr ps
 G S 53
 Kemp W J 554
 Kempster H L see Hlog n
 A G 523
 Kennard D C see Bethk R
 M 418
 Kennedy T and Sprng F S
 399
 Képinov L 91 460
 Keppel D M see Vigneaud V
 du 543 546 547
 Keresztessy J C and Stevens
 J P 198 199 211
 Keresztessy J C Steven J P
 Harris S A Stiller E T
 and Folkers K 198
 Keresztessy J C see Scudi
 J V 199 210 212 213
 Keresztessy J C see Stiller
 E T 200-20 255 257 259
 261 264 271
 Keresztessy J C see Williams
 R R 101 104 119 125
 Kern R Montgomery M F,
 and Still E W 380
 Kerns W M 92
 Kersten G and Schultz O A
 60
 Kertess Z I Dearborn R B
 and Mack G L 327
 Key K M and Elphick G K
 323
 Key K M see Cow rd K H
 415 420
 Kh rasch M S and Wein
 house S 595
 Kharasch M S see Weinhouse
 S 363
 Kh ustov N W see Charite
 A J 18
 Kielwein F 60
 Kielwein F see Wörner A 597
 Kik M C see Sure B 89
 Kill fter D H 61
 Kimble M S and Gordon
 E S 192 333
 Kimmig J 285
 King C G 328 546
 King C G and Menten M L
 330 333
 King C G and Waugh W A
 587
 King C G see Ha rer C J
 3 9
 King C G see Longenecker
 H E 313
 King C G see Lym n C M
 331
 King C G see Musulin R R
 312
 King C G see Sigal A 330-
 333
 King C G see Stotz E 3 7
 King C G see Wa gh W A
 291 293
 King J D see M llanby M
 4 8
 Kinnersley H W O'Brien
 J R and Peters R A 101-
 104 120 124 128 130 131
 Kinnersley H W Peters R
 A and Reader V 183
 Kinnersley H W see Carter
 C W 5 2
 Kinnersley H W see Jansen
 B C P 102
 Kinter J H and Holt R L
 437
 Kipp n H see Reindel F
 353
 Kipping F B and Wild F
 65
 K. by G W and Grey C A
 556
 Kirchmeyer P J see Moor
 M B 494
 Kirk C H see Bethke R M
 418 419
 Kutiakowsky G B see Cr mer
 R D 542
 Kitasato T see N uberg C
 306
 Kituta K 571
 Klaverson F W von see Kuhn
 R 120
 Kleiderer E C and Rhode-
 hamel H W 59
 Klein D 148
 Klein J R 230
 Klein J R and Kohn H I
 184 187 192 233 244
 Klein M 561
 Klein S 591
 Kleiner I S see Tauber H
 322 335
 Kleinseller A see Krebs H A
 137
 Klemperer F W see Conant
 J B 542
 Klemperer F W see Jandorf
 B J 234
 Klemperer F W see Solomon
 A K 54
 Klink E 538
 Klenk E and Dittmar J 532
 Klenk E and Schoenebeck O
 von 532
 Kletzien S W F see Ste n
 bock H 385
 Klewan G see Josephson E
 M 247
 Kline A A see Lampen J O
 470 474
 Kline C L see Arnold A
 541
 Kline J see Major R T 611
 Kline O L Bird H R
 Elvehjem C A and Hart
 E B 572
 Kline O L Elvehjem C A
 and Hart E B 521
 Kline O L Tolle C D and
 Nelson E M 130
 Kline O L see Ke n n J A
 571
 Klingensuss M 116 576
 Klink K 424
 Klose A A and Almqvist
 H J 483
 Klose A A see Almqvist
 H J 48 485 492 494 497
 499 501 502
 Klotz A W see Mueller J H
 264
 Klusmann E see Euler H v
 ol 56 69 79 91 290
 Knapp A W and Coward
 K H 385
 Knight B C J G 134 220
 242 243 250
 Knight B C J G and Mc-
 llwain H 242
 Knight C A Dutcher R A
 and Guerrant N B 292

- Knöfel A F see Topping
M C 192
- Knott E M 150
- Knowles H R Hart E B and
Halpin J G 406
- Knowlton C C and Hines
H M 46⁹
- Knox W F see Green D F
171
- Knudson A 598
- Knudson A and Benford F
369
- Knudson A and Floody R J
418
- Knudson A and Moore C N
371
- Knudson A see Marshall A
L 368
- Krunyantz I J and Katz
nelson M M 586
- Knutson M H see Bechdel
S I 101 150
- Kobayashi E and Yamamoto
A 80
- Koch E M and Koch F C
362
- Koch E M and Lemon H B
362
- Koch E M see Koch F C
362
- Koch F C Koch E M and
Ragins J A 36⁹
- Koch F C see Koch E M
362
- Kodicek E 221 241
- Kögel R 277
- Kögl F 469 470
- Kögl F and Fries N 134
279
- Kögl F and Haagen Smith
A J 133 470 475
- Kögl F and Hasselt W van
276 470
- Kögl F and Man T J de
472
- Kögl F and Pons L 472
- Kögl F and Tonnus B 471
474
- Kögl F and Wagtenonk W
J van 475
- Koehn C J see Elvehjem C
A 156 242 253 254 469
- Koelsch C F and Byers D J
497
- Koenig H see Karrer P 44⁹
445 449
- König W 240
- Körner F 611
- Köser J see Windaus A 381
- Köthning M see Brederick H
170
- Kogut B see Zuckerman I C
507
- Kohler G O Elvehjem C A
and Hart E B 526
- Kohler G O Randle S B
Elvehjem C A and Hart
E B 526
- Kohler G O Randle S B and
Wagner J R 597
- Kohler C O see Randle S B
596
- Kohman E I Lddy W H
and Gurin C Z 312
- Kohn H I 299 234
- Kohn H I and Klein J R
933
- Kohn H J Klein J R and
Dann W J 244
- Kohn H I see Dann W J
250
- Kohn H I see Klein J R 184
187 192
- Kohn M and Neustädter V
261
- Kollath W and Magstris H
558
- Koller F 509
- Koller F see Fenthardt F
328
- Komm E 555
- Kon S K Daniels F and
Steenbock H 362 368 409
- Kon S K see Campion J E
403 406
- Kon S K see Houston J
101 130 147
- Koonen H F see Scudi J V
199 210 212 215
- Korenchevsky V 342 421
- Korenschewsky V see Copping
A M 408 46⁹
- Korselt J 558 593
- Koschara W 187
- Koschara W see Ellinger P
154 156 188
- Koschorreck K see Widen
bauer F 312
- Koser S A Dorfman A and
Saunders F 243
- Koser S A see Dorfman A
246 249
- Koser S A see Saunders F
246
- Kosel A J see Fohmann K
123
- Krämer K see Ott E 301
315 329
- Kraft K see Michael I 291
297 299 300 305
- Kramer B
Kramer B and Sobel A E
599
- Kramer B and Tisdall P F
429
- Kramer B see Howland J
420
- Krause A C and Weekers R
276
- Krause M E see Lepkovsky
S 197 253 254 257 269 270
477
- Krauskopf E J Snell E E
and McCoy E 185 267 270
- Krauss W C see Bethke R
M 418
- Kraut H see Iantschenko
Jurewicz W v 399
- Krebs H A 138 140 175
- Krebs H A and Cohen P P
228
- Krebs H A and Eggleston
L V 136 138 139 141
- Krebs H A Eggleston L V
Knechtler A and Smyth
D H 137
- Krebs H A and Johnson
W A 137
- Krenn L 417
- Kretschmer E 556
- Kreuchen K H 404
- Kringstad H and Lunde G
472
- Kringstad H and Naess T 240
- Kringstad H see Lunde G
198 199 211 214 253 283
469 470 473
- Krishnamurthy P V see Gini
K V 327
- Krönig H 559 560
- Kropp W Lange F and
Bohne A 555 560
- Kruse H D Sydenstricker
V P Sebrill W H and
Cleckley H M 191
- Kubli U see Karrer P 128
- Kubowitz F 377
- Kudrjashov B A 457
- Kühling O 163
- Kühling O and Kasselitz O 163
- Kühnau J see Stepp W 193
212
- Kugel V H 283
- Kugelmass I N 517 518
- Kuhn R 193 570 581
- Kuhn R and co-workers 41
- Kuhn R Andersag H West
phal K and Wendt G 202
203
- Kuhn R and Biehl H J 39
43 50
- Kuhn R and Boulanger P
160 181 193
- Kuhn R and Brockmann H
38-41 44 53 74 75 80 96
- Kuhn R Brockmann H
Scheunert A and Schieblch
M 74
- Kuhn R and Cook A H 164
- Kuhn R and Desnuelle P 6
172
- Kuhn R and Grundmann C
39 41 571
- Kuhn R György P and
Wagner Jauregg T 154 156
160
- Kuhn R and Kaltschmitt H
155 189
- Kuhn R Kaltschmitt H and
Wagner Jauregg T 187 188
192
- Kuhn R and Karrer P 154
- Kuhn R and Lederer E 38 44
- Kuhn R and Löw I 210 213

- Kuhn R and Möller E F 392
 Kuhn R and Morris C J O R 37 63 571 572
 Kuhn R and Moruzzi G 157 160
 Kuhn R and Reinemund K 163
 Kuhn R Reinemund K and Weygand F 163
 Kuhn R Reinemund K Weygand F and Stroebel R 166 170
 Kuhn R and Rudy H 157 159 172 177 179 184 583
 Kuhn R Rudy H and Reinemund K 163
 Kuhn R Rudy H and Weygand F 158
 Kuhn R Rudy H and Weygand F 158 177
 Kuhn R and Ströbele R 161 166 167 58
 Kuhn R and Tecklenburg M L 73
 Kuhn R and Vetter H 120 126 221 229
 Kuhn R Vetter H and Rzeppa H W 181
 Kuhn R and Wagner Jauregg T 18 159
 Kuhn R Wagner Jauregg T and Kaltschmitt H 183
 Kuhn R Wagner Jauregg T Klavert F W von and Vetter H 190
 Kuhn R and Wallenfels K 572
 Kuhn R Wallenfels K Weygand F Moll T and Hepding L 497
 Kuhn R and Wendt G 198-203 209 211 13 214 16 255 584
 Kuhn R Wendt G and Wepphal K 09
 Kuhn R Westphal K Wendt G and Westphal O 198 206 707
 Kuhn R and Weygand F 164
 Kuhn R Weygand F and Rudy H 582
 Kuhn R and Wieland T 255 256 260 264-267
 Kuhn R Wieland T and Huebschmann H 142
 Kuhn R and Winterstein A 46 50
 Kuhn R see György P 154 155 183 197 470 471 574
 Kuhn R see Lechmeister L 37
 Kuhr E see Windaus A 409
 L
 Labbé H 603
 Laborey F see Lavollay J 185 189
 Lachat L L 283
 Ladenburg A 224
 Ladisch R K see Spates T D 215
 LaForge F B 225
 Lagerlöf N 461
 Laible R 224
 Laine T see Virtanen A I 138 263
 Lajos S 518
 Laki L Straub F B and Szent György A 138
 Lampen J O Bahler G P and Peterson W H 470
 Lampen J O Kline A A and Peterson W H 470 474
 Lampen J O and Peterson W H 86
 Lampen J O see Hottle G A 475
 Lampitt I H and Bushill J H 565
 Lampman C I see Williams J K 96
 Landfisch S 330
 Landy M 242 243
 Landy M and Dicken D M 475
 Landy M and Wyeno J 985
 Landy M see Ausbacher S 474
 Langford C S see Sherman H C 186
 Lang K and Mayer H 573
 Lange A E 463
 Lange F and Taube L 573
 Lange F see Kropp W 555 560
 Langer S Windus A 344 349 363
 Langfeldt E and Hellerud R 562 565
 Langlois G A and Deloson A M 586
 Langston W C Darby W J Shukers C I and Day P L 54
 Langston W C see Day I I 183 191 524
 Languz A see Berich R 160
 Lanman T H and Ingalls T H 333
 Lantz P M 155 186 199 212
 Lanzing J C see Veen A G v n 55
 Lauer F 560 570
 Laquer F and Linsert O 380
 Lauer F see Windus A 100 109
 Lasky A W see From V C 601
 Larson R see Arnold R T 491
 Lasch F and Roller D 85
 Lassen S H 580
 Laszt I 247 250
 Laszt L see Verzar F 186 188 190
 Latacz E 609
 Latacz C see Querido A 249
 Lauber H J and Rocholl H 84
 Laufer E see Tauber H 284
 Lautenschläger C L and Lindner 584 585 588 592 593 606
 Lavollay J and Laborey F 18, 189
 Lawso W F and Spaeth C P 58
 Lea C J G 471 473
 Lea J G and Parsons H T 469 471 473
 Lease J G Parsons H T and Kelly E 477 478
 Leblond C P 322
 Leblond C P see Giroud A 330
 Lecoq R 409
 Lecoq R and Duffan R 409
 Lederer E 42 87
 Lederer E and Edisbury J R 37
 Lederer F and Moore T 42
 Lederer E and Rathman F H 71
 Lederer E and Roanova V 68
 Lederer E see Gillam A E 59 68 70
 Lederer E see György P 470 471
 Lederer E see Kuhn R 38 44
 Le J see Foster R H K 494 499
 Lee H see Gdeman N T 416
 Legler R C see Karr R P 450 483
 Lehmke H 463
 Leichter H M see Holmes H N 569
 Lezrovkaya M J see Mikhlina E J 384
 Leloir L F see Green D E 228
 Lemberg R Cortis Jones B and Norri M 328
 Lemon H B see Koch E M 36
 Lenstrup C see Iverson P 420
 Lenthardt F and Koller F 378
 Lentz R W 463
 Leong P C see Harris L J 147
 Lepkovsky S 197 198
 Lepkovsky S and Jukta T H 191

- Lepkovsky S Jukes T H and Krause M E 197 203 254 267 269 270 477
- Lepkovsky S Popper W and Evans H M 156
- Lepkovsky S Taylor L W Jukes T H and Almquist H J 188 191
- Lepkovsky S see Evans H M 531
- Lepkovsky S see Iouts P J 214 220 254
- Lepkovsky S see Jukes T H 203 266
- Lepp A see Dimick M K 263
- Léranth G and Frank I 408
- Leser A J Lombard C I Thuenes C H Wawra C and Webb J I 117
- Leh J B Underkoffler I A and Lulmer L I 278
- Leselberg W 560
- Lettré H 375 378 393
- Lettré H see Windaus A 344 360
- Levene P A 102
- Levene P A and Rolf I P 32
- Levin I see Weisberg S M 189
- Levine J and Steinhaus A H 404
- Lewis J M 410
- Lewis J M Bodansky O Falk K F and McGuire G 80
- Lewis J M see Hess A F 410
- Lewis L see Dam H 483 506
- Lexow T B 566
- Liebermann C 367
- Liebers H 554
- Liebig J v 9 11 275 469
- Lieck H see Lund H 310 330
- Lienz M see Eisenbrand J 591
- Light R F and Frey C N 573 594 598
- Light R F Miller G and Frey C N 426
- Light R F Wilson I T and Frey C N 419
- Light R F see Frey C N 594
- Light R F see Hess A F 418 419
- Lightbody H D see Huston R C 563
- Lilienfeld A Wright I S and MacLenathen E 326
- Lilienfeld A see Wright I S 375 336
- Lillie R D Daft F S and Sebrell W H 544
- Lillie R D and Goldberger J 198 220 242 254 269 477
- Lillie R D see Daft F S 544
- Lind 8
- Lindenfeld K 276
- Lindner A W see Harrel C G 556
- Indholm H 183
- Indner F see Fischer H 398
- Indner F see Lautenschläger C I 584 585 588 59 593 606
- Indner H see Heiduchka A 300 367
- Indner P 566
- Ingane J J and Davis O I 183
- Inn D R see Shepherd M I 337
- Instal see Windaus A 341 364 407
- Insert O 344 360 604 606
- Insert O see Iaquer F 380
- Insert O see Windaus A 344 360 361 362 374 387
- Lipmann F 140 144 298
- Lippincott S W see Morris H P 269
- Lipschitz M A Van Potter R and Elvehjem C A 124
- Lipshutz M D 462
- Lipton M A and Elvehjem C A 125
- Lipton M A see Barron E S G 139 141
- Lipton M A see Mannering G J 188
- Lipton M A see Sober H A 138
- Lipton M A see Wagner J R 183
- Iischer C F see Martin G J 497 499
- Ioach J V see Beeston A W 545
- Loach J V see Channon H J 548
- Lobb D E see Hathaway M L 362
- Locher F see Gams A 594
- Lockhart F E 173
- Lockwood L B see Wells P A 304
- Löw I see Kuhn R 210 213
- Löwenberg K see Fischer F G 488
- Löwenthal L J A 92
- Lohmann K 125
- Lohmann K and Kossel A J 123
- Lohmann K and Schuster P 100 123 124 126 136 146 380
- Lohmann W see Fries K 490
- Lohmann W see Michael P 305
- Lohr H see Mohler H 204 295 316
- Lombard C F see Leser A J 517
- Long C 140
- Long C and Peters R A 136
- Longenecker H E Gavin G and McHenry F W 142
- Longenecker H F Musula R R Tully R H and King L C 313
- Longenecker H I see Musula R R 312
- Loofbourow J R see Heyroth F F 100
- Loomis H I 513
- Lord J W see Andrus W D 506
- Lozt J see Kerner P 477
- Lothrop W C see Anderson R J 78
- Lotze H 597
- Lourenço J A de 571
- Lovern J A Mead T H and Morton R A 70
- Lovern J A and Morton R A 71
- Lovern J A Morton R A and Ireland J 70
- Lovern J A see Edisbury J R 58 71
- Lovett Jansson P L and Nelson J M 327
- Lowell F C see Strauss E 286
- Loy H W see Scott H M 410
- Lozner E L Poble F J and Taylor F H L 325
- Lozner E L see Kark R 507 509
- Lozza M 333
- Lu C D 148 149
- Lu G D and Platt B S 136
- Lu G D see Platt B S 136 148
- Lubarsky G H 565
- Luccardi G 427
- Lucas G H W 275 469
- Lucas N S see Hume E M 417
- Lucia S P see Aggeler P M 506 507
- Ludwig F 517
- Lüttrichhaus A see Windaus A 344 350 354 356 374 377 380 387
- Lund H 335 336
- Lund H and Lieck H 319 335
- Lunde G and Kringstad H 198 199 211 214 253 283 469 470 473
- Lunde G Kringstad H and Jansen E 253

- Lunde G see Kringstad H 472
 Lunin 9-11
 Just 425
 Lutwak Mann C 228
 Lutwak Mann C see Dixon M 28
 Lwoff A and Dust H 134
 Lwoff A and Lwoff M 229 234 250
 Lwoff A and Querido A 242
 Lwoff A Querido A Dixon net L and Garmier 246
 Lwoff A see Querido A 249
 Lwoff M 325
 Lwoff M see Lwoff A 20
 Lyman C M and King C G 331
 Lynn C M see Bron E S G 138 141 144
 Lyman C M e Williams R J 33-9 27 266
 Lynn M e Yoder L 409
 Lythgoe B e El Sadr M M 264
 Lythgoe B see Heilbron I M 42
 Lythgoe R J 90
 Lythgoe R J and Tan ley K 90

 M
 Mas G J van der see Dols M J I 423
 McAister F D e Williams R J 260
 McArthur C S and Watson E M 456
 MacBryde M 380
 MacBurney C H Bollen W B and Will ms R J 255 270
 MacBurney C H see Williams R J 255 257 266
 McCann D C see Olcott H S 54 56
 McCarter J R e Woolley D W 504 509
 McCarty M 285
 McCay C M Bing F C and Duley W E 197
 McCay C M see Bowers R E 4 95
 McClement J see Davis J G 324
 McCollum E V 13 343
 McCollum E V and Davis M 12 37 57
 McCollum F V and Simmonds N 34 421
 McCollum F V Simmonds N Shipley F G and Parks E A 343 414 41
 McCollum F V e Blumberg H 544
 McCollum E V see Itter S L
 McCollum E V see Macken zie C G 454 458 462 464 465
 McCollum E V see Prebluda H J 129 130
 McCollum E V see Shils M 148
 McCoard A B see Chesney J 95
 McCoard A B see Clausen S W 89 94
 MacCorquodale D W Binkley S B Thayer S A and Doisy E A 487
 MacCorquodale D W Cheney L C Binkley S B Holcomb W F McKee R W Thayer S A and Doisy E A 493
 MacCorquodale D W see Binkley S B 482 484 486 487 489 493 499
 MacCorquodale D W see Doisy E A 494 499
 MacCorquodale D W see McKee R W 482 484 486 489 496
 MacCorquodale D W see Thayer S A 502-504
 McCoy E see Krauskopf E J 185 267 270
 McCoy E see Peterson W H 475
 McCreary J F see May C D 56 93 94
 MacCurdy J T see Mapson L W 559
 McDaniel I E see Peterson W H 475
 Macdonald C A see Shaw G E 240
 McDonald F G 407
 McDonald F G see Bill C E 240 409
 McFroy L W and Go H 10 194 21 216 266 271
 McElvain S M and Goe M A 225
 McFarlan R I and Redde J W 572
 McFarlan R I Redde J W and Merrill L C 78
 McFarlane W D see Park r W C 4 3
 McFetridge E M see Boyce F F 508
 McFey G H McLean A L Spindle F and Maxey K F 59
 McGowan G K and Peters R A 138
 McGue G see Lewis J M 80
 McHenry E W 142
 McHenry E W and Gavin C 142 188 213 476
 McHenry E W and Graham M L 99 301 323
 McHenry E W see Best C H 543
 McHenry E W see Gavin G 280
 McHenry F W see Longe necker H E 142
 McHenry E W see Reedman E J 9 301 39
 Macht D I see Anderson W T 403
 Mellwain H see Knight B C J G 249
 McIntyre A R see Burke J C 145
 Mack G L see Hertesz Z 1 327
 Mackay see Farli 427
 McKee R W Binkley S B MacCorquodale D W Thayer S A and Doisy F A 487 488 489 496
 McKee R W Binkley S B Thayer S A MacCorquodale D W and Doisy E A 484
 McKee R W see Binkley S B 48 484-490 493 499
 McKee R W see Doisy E A 494 499
 McKee R W see MacCorquodale D W 493
 McKee R W see Richtert D 494 499
 McKee R W see Thayer S A 502 504
 Mackenzie C G and McCollum E V 454 458 462 464 465
 McKibbin I M W see Cap per N S 55
 McKibbin J M see W man H A 240
 McKibbin J M Black S and Elvehjem C A 270
 McKibbin J M Madden R J Black S and Elvehjem C A 214
 McKin I H see Emmett A D 154 521
 McKinly W A e Bak n R I 476
 Mackintosh J s Camp on J E 403 406
 McLan A L see McG nnes G F 9
 MacLean D L R dout J H and Best C H 545
 McLean I S 600
 MacLennan E see I ienfeld A 376
 McLennan E see Wright I S 33
 MacPhilly H B 360
 MacPhilly H B see Ansbacher S 48
 MacPhilly H B see Fernholz E 496 03

- McQuarrie I Thompson W
H Stoesser A V and Rig
ler L G 372
- McQuarrie I see Streser
A V 544
- Macrae T F and Edgar C E
52^o
- Macrae T F see Chick H
214 250
- Macrae T F see Edgar C E
197 198 211 214 253 266 269
- Macrae T F see El Sadr
M M 183 197 264
- MacWalter R J see Drum
mond J C 438 440
- Macy I G see Hunscher H
A 430
- Macy I G see Slyker F 4^o
- Madden R J see Axelrod
A E 244
- Madden R J see Elvehjem
C A 220 242 250
- Madden R J see McElbbsin
J M 214
- Madden R J see Woolley D
W 239
- Madunaveita J 490
- Magath T B 508
- Magath T B see Snell A M
507
- Magistris H see Kollath W
558
- Maguigan W H and Walker
E 365 366
- Mai H 371 404 406
- Mauzel B 574
- Major J 603
- Major R T and Cook E W
590 591
- Major R T and Finkelstein
J 262
- Major R T and Kline J
611
- Major R T see Jones L W
611
- Major R T see Williams R J
259
- Majumdar B N 84
- Makino K and Imai T 113
- Makino K see Imai T 107
- Makino K see Mori S 204
- Malik K see Ahmad B 55
- Malloy H T see Evelyn K A
310
- Malmberg M see Euler H v
76 167 181 186 220 30
325 523
- Malmberg M see Karrer P
181
- Maltha P R A 593
- Man T J de see Kogi F
472
- Manchester T C 554
- Mandelbaum J see Hecht S
90 93
- Manifold M C see Channon
H J 545
- Mann T see Keilm D 327
- Mannerling G J Lipton M A
and Elvehjem C A 188
- Manning P D V see Almquist
H J 542
- Manning P D V see Stokstad
E L R 191 525 54^o 543
- Mapson L W MacCurdy J
T Nolan H O 559
- Mapson I W see Birch T W
123
- Maquenne 276
- Marcus J K 554
- Marcussen E 412
- Margolis G see Margolis L
H 102
- Margolis L H Margolis G
and Smith S G 192 247
- Margolis L H see Margolis
G 747
- Marker R F 595
- Marmorston J see Perla D
331 333
- Marquardt P see Hoffman F
591
- Marrack J and Höllering H
F 147
- Marshall A L and Knudson
A 368
- Marshall W 469 477
- Martin A J P and Moore T,
461
- Martin A J P see Chick H
214 250
- Martin A J P see Moore T
437 456
- Martin G J 285
- Martin G J and Ansbacher S
280 285 286
- Martin G J and Lischer C F
497 499
- Martin G J Tchrowski C T
Wisansky W A and Ans
bacher S 285
- Martin G J Thompson M R
and Carvajal Forero J de
245 279
- Martin G J Wisansky W A
and Ansbacher S 285
- Martin G J see Chick H
250
- Martin G J see Wisansky
W A 285 286
- Martin R W 190
- Martini E and Bonignore A
30
- Martius C see Euler H v
290 317
- Martius H 463
- Maschmann E 228 328
- Mason K E 458 461
- Mason K E see Simpson
J W 9^o
- Massart L and Dufait R 14^o
143
- Massengale O N and Bills
C E 415 416
- Massengale O N Bills C E
and Prickett P S 366
- Massengale O N and Nuss
meier M 344
- Massengale O N see Bills
C E 344 384 401 411
- Matheron R see Chevallier
A 94
- Mathews A P see Vilter
S P 241 248
- Matill H A and Conklin
R E 436
- Matill H A see Olcott H S
437 438 440 454 462
- Matsunaga S see Suzuki W
220
- Matukawa T and Ohta M
576
- Maurer A and Schiedt B 314
- Mayer M E and Voegtlin C
328
- Maxcy K F see McGinnes
G F 545
- May C D Blackfan K D
McCreary J F and Allen
F H 56 93 94
- May E L see Westminster H
H 265
- Maybury E 558
- Mayer H see Lang K 523
- Maynard L A see Williams
H H 539
- Mead T H 62
- Mead T H see Lovern J A
70
- Mead T H Underhill S W F
and Coward K H 81
- Mecchi E see Almquist H J
483 494 542
- Mecchi E see Stokstad E L
R 543
- Meek W J see Jacobi H P
347
- Meerwein H see Karrer P
165 177 179
- Meiklejohn A P 131
- Meinerts U see Butenandt A
407 408
- Mellanby E 34 471
- Mellanby M and King J D
428
- Melmick D and Field H 124
129 148 140 248 249
- Melmick D Robinson W D
and Field H 241 248 249
- Melmick J L see Stern K G
144
- Meltzer M 92
- Melville D B see Carter H
E 545
- Melville D B see György P
470 471 474
- Melville D B see Hofmann
K 47^o
- Melville D B Hofmann K
and Vigneaud V du 472
- Melville D B see Vigneaud
V du 470-472
- Melzer L see Euler H v 221
222 241 250 54^o

- Mendel L B see Osborne
T B 12 37 154
- Menten M L see King C G
330 333
- Mentzer C and Urbain G
312
- Mecker H M 563
- Merrill E C see McFarlan
R L 78
- Mettler S R see Borson H J
214
- Metz G A see Coppens P A
385
- Metzger N see Baumann
E J 293
- Meunier P and Blancpain
C P 199
- Meunier P Hinglais H
Bovet D and Dreyfuss A
503 509
- Meunier P see Raoul Y 79
- Meyer A 330
- Meyer A E 555
- Meyer C E see Smith A H
138
- Meyer C E see Williams
R J 255 267
- Meyer H see Rominger E
429
- Meyer J see Sartory A 34
- Meyer R E see Furter M
453
- Meyerhof O 230
- Meyerhof O and Ohlmeyer
P 229
- Meyers H C 561
- Michaels L 229
- Michael S L and Schwaben
B C G 160
- Michaelis I see Runnström
J 232 36
- Michael M see Adler E
174
- Michael F and Haerhoff H
311
- Michael F and Kraft K 291
297 299 300 305
- Michael F and Lohmann W
305
- Michael F and Mittag R
313
- Michael F and Moll T 314
- Michael F Rühkopf H and
Suckfull F 78
- Michael O see Waisman
H A 240 255
- Michael O Waisman H A
and Elvehjem C A 253 270
- Michael O see Woolly
D W 3 257 264 270
- Mieg W see Willstätter R
46 50
- Miescher K. and Tschopp E
20
- Miyata T see Fujita A 30
- Mikhail J Lezerovskaya
M J and Milovanova N N
384
- Milas N A 563 599
- Milas N A and Alderson W
L 403
- Milas N A and Heggie R
382
- Miles E H and Penley G 555
- Mille C see Verne J 572
- Miller 469 470
- Miller D K. and Rhoades
C P 334
- Miller G see Light R F
426
- Miller H 92
- Miller R F see Guilbert
H R 90
- Miller S E 604
- Mills C A 34
- Mills C S see Townsend
S T 507
- Mills J I see Harris L J
323
- Mills R C see Hegsted D M
474 477 545
- Moll W H Clark L M and
Aeschmann J A 114
- Milovanova N N see Mikhail
F J 384
- Mindlin R J and Butler
A M 318 319 335
- Miner C S 574
- Minnberg N 553
- Minor F W see Allison F W
474
- Mintz B 14
- Mintz B and Agid R 140
- Mitchell H H see Hamilton
T S 183
- Mitchell H K 586
- Mitchell H K. see Weinstock
H H 258 264 267
- Mitchell H K see Williams
R J 255 257 260
- Mitchell H K. Snell E F and
Williams R J 263 264 520
- Mitchell H K. Weinstock
H H Snell E E Stanbery
S R and Williams R J
256 258 265
- Mits K. see Itaba A 198 199
01 202 206 207
- Mittag R see Michael F 313
- Mittag H 34
- Möller E F 523
- Möller E F Fma O Jung
P and Moll T 209 211
- Möller E F see Kuhn R
392
- Möller E F see Wagner
Jurg T 174
- Moewus F 87
- Moggridge R C G and
Ogden A G 113
- Moggridge R C G see Harug
ton C R 119
- Mohammad A see Emerson
G A 199
- Mohler H and Lohr H 294
295 316
- Mohr H 150
- Molitor H and Robinson
H J 509
- Molitor H and Sampson W
L 104
- Moll T 516
- Moll T see Dalmer O 314
387
- Moll T see Kuhn R 497
- Moll T see Michael F 314
- Moll T see Miller E F 209
211
- Moll T see Werde F v 449-
451
- Moll T see Zima O 143
- Mollgaard H 96
- Molotsky D 562
- Moness F see Christensen
W G 56
- Montgomery M F see Kern
R 380
- Moor 37 83
- Moore B see Edie C S 99
- Moore B see Strong F M
184 187 192
- Moore C N see Knudson A
371
- Moore E E 586
- Moore I A 92
- Moore M B 494 586
- Moore M B and Kirchmeyer
F J 494
- Moore M B see Volwiler
E H 591
- Moore M L see Ansbacher
S 610
- Moore M L see Fernholz E
48
- Moore R A see Andrus W D
206
- Moore R B and DeVries T
371
- Moore R 54 59 78-80 91
460
- Moore T Martin A J P and
Rajagopal K R 437 456
- Moore T and Rajagopal K
R 451
- Moore T see Baumann C H
91
- Moore T see Davis A W 86
01
- Moore T W see Harris L J
380 46
- Moore T see Leder E 40
- Moore T see Martin A J P
461
- Morawitz P 500
- Morl A see Arlong F 593
- Morrell J 462
- Morf R see Euler H v 79
- Morf R see Karr P 54 60
61 63 65 96 298
- Morgan A F 431
- Morgan A F Cook B B and
Davison H G 253
- Morgan A F Hendricks J B
and Freytag R M 430

- Morgan A F and Simms
 H D 253
 Morgan F J see Hopkins
 F G 327
 Morgan G F see Coward
 K H 415 420
 Morgan R S 79
 Morgareidge K see Gray F
 L 85
 Morgareidge K see O'Brien
 B 408
 Morgulis S 462
 Morgulis S and Spencer H C
 458
 Moril S and Makino K
 204
 Moro E 476
 Morris C J O R see Kuhn
 R 37 65 571 572
 Morris H P and Lippincott
 S W 269
 Morris N and Peden O D
 4 9
 Morris N and Stevenson M
 M 412
 Morris N Stevenson M M
 Peden O D and Small
 J M D 424
 Morris S D D see Bowden
 F P 81
 Morris S G 462
 Morrison A L see Heilbron
 I M 354
 Morse M see Schlutz F W
 343
 Morton R A 72 74 80 316
 Morton R A and Creed R H
 55 70
 Morton R A and Heilbron I
 M 59
 Morton R A see Coward K
 H 79 80
 Morton R A see Edisbury
 J R 58 62 68 71
 Morton R A see Gillam A E
 79
 Morton R A see Heilbron I
 M 60-63 72 79 343
 Morton R A see Lovren J A
 70 71
 Morton R A see Pritchard H
 63 72
 Moruzzi G see Kuhn R
 157
 Mosely R L see Burr G O
 462
 Moser R see Williams R J
 255 257
 Mosher L M see Bunker J
 W M 369
 Mosher L M see Harris R S
 363
 Mosher W A see Williams
 R J 267
 Mosonyi J 313
 Mosonyi J and Rigo L 328
 Moss A R and Drummond
 J C 437 438
 Mouriquand G 32
 Mouriquand G Dauvergne
 M and Edel V 326 337
 Moussu R 463
 Mowry D T Brode W R
 and Brown J B 535
 Moyer A W see Vigneaud
 V du 543 546 547
 Mozolowski W 347
 Mueller D 177
 Müller E 429
 Müller H and Reichstein T
 314
 Mueller J H 220 243 250
 263 264
 Mueller J H and Klotz A W
 264
 Müller M 379 382 397 399
 Mulford D J see Griffith
 W H 544 545
 Mullen J W see Pacsu E
 262
 Muller J and Hoffman U 590
 Muller P see Bergh A A H
 van den 38
 Munsell H E 183
 Munsell H C see Daniel E P
 16
 Munsell H E see DeVaney
 G M 406 419
 Munsey V E 53
 Murphy E A see Evans H
 M 531
 Murphy R 509
 Murthy G N 182
 Musher S 572
 Musschl F B and Ackerson
 C W 344
 Musulin R R Tully R H
 Longenecker H E and
 King C G 312
 Musulin R R see Longenecker
 H E 313
 Muus J see Hastings A B
 190
 Myers R P and Weisberg S
 M 556
 Myrbäck K 231 232 234
 Myrbäck K Euler H v and
 Hellstrom H 230 231 236
 Myrbäck K and Larsson H
 230
 Myrbäck K and Örtenblad B
 231
 Myrbäck K see Euler H v
 222 232 234
- N
- Naess T see Kringstad H
 240
 Nagatz J see Windaus A
 349 363
 Nauman B 129
 Nakahara W and Inukai B
 581
 Nakahara W Inukai F and
 Ugami S 524
 Nakamiya Z 63
 Nakamiya Z see Guteras A
 375 393
 Narayanan B T and Drum
 mond J C 15
 Nath M C see Basu K P
 317
 Nedzvedsky V A see Taylor
 H F 564
 Needham D M see Green
 D E 228
 Negelein E and Brömel H
 175 184
 Negelein E and Gerscher W
 228
 Negelein E and Wulff H J
 228
 Nelson E A and Browne C
 A 317
 Nelson E K and Keenan G
 I 276
 Nelson E M see Ehot M M
 412
 Nelson E M see Kline O L
 130
 Nelson E M see Sternbock
 H 53
 Nelson J M see Lovett
 Janison P L 327
 Nelson J M see Taylor T C
 532
 Nelson M T see Steenbock
 H 343
 Nelson W O 459
 Neracher O and Reichstein T
 387
 Netter R 38
 Netter R see Bailly O 38
 Neuberg C 226
 Neuberg C and Kitasato T
 306
 Neugebauer R 559
 Neumann H O 334
 Neustädter V see Kohn M
 261
 Neuweiler W 513
 Newman M S see Anderson
 R J 490
 Newton R C see Robinson
 H E 254 269 477
 Ney L F see Carter H E
 261
 Nicolaysen R 427
 Nicolaysen R and Jansen J
 422
 Niederberger W see Gander J
 336
 Niederberger W see Jezler A
 336
 Niederländer K see Reindel
 F 356 366
 Niekerk J van and Bhek M
 S C 419
 Niekerk J van and Franken
 F 407 409
 Niekerk J van and Hof tra
 419

- Nekerk J van see Boer A G
344 346 349 359 367 407
595
- Nield C H Russell W C
and Zimmerli A 413
- Nilsen L and Lihjem C A
477
- Nir A O see Wood H G
139 141
- Nightingale E see Andersen
A 78
- Nilsson R 20
- Nilsen R Bjälve G and
Burstrom D 475
- Nims V see Day P L 54
- Nitardy F W 555 557 562
564
- Nitardy F W and Jones W
S 563
- Nitschke A 414 496 497
- Nitti F see Trélonet J O
- Noble R L see Drummond J
C 460
- Nolan H O 562
- Nolan H O see Mapson L W
559
- Norbin A B O 555
- Norbin A B O and Astra A
558
- Norris M see Lemberg R
378
- Norris E R and co-workers
79
- Norris E R and Church A B
79 426
- Norris L C and Ringrose A
T 220 246 254 269
- Norris L C Wilgus H S
Ringrose A T Heiman V
and Heuser G F 185
- Norris L C see Bauernfeind
J C 253 269 270
- Norris L C see Davis H J
191 194
- Norris L C see Heuser G F
191 194
- Norris L C see Hodson A Z
118
- Norris L C see Ringrose A
T 254 269 478
- Norris L C see Wilgus H S
63
- Norris R J see Spertl G 598
- Notewark O 82
- Novas J L 92
- Novik A. 501
- Nukida Z 594
- Nunn L C A and Smedley
MacLean I 531 535 538
- Nunn L C A see Hume E
M 536 537 539
- Nunn L C A see Smedley
MacLean I 538
- Nussmeier M see Massengale
O N 344
- Nygaard K K 503
- Nygaard K K see Guthe T
319
- Nyrop A 559
- Nyrop J E 558 559
- O
- O'Brien B and Morgareidge
K 408
- O'Brien C. S see Day P L
191
- O'Brien J R see Carter C W
114 253 521 522
- O'Brien J R see Kinnrley
H W 101-104 120 131
- Ochoa C G G see Ocho S
229
- Ochoa S and Ochoa C G
229
- Ochoa S and Peters R A
101 132 135 146 149
- Ochoa S and Rossiter R J
184 188 192
- Odake S see Suzuki U 99
- Oden J W Oden L H and
Sebrell W H 191
- Oden L H see Oden J W
191
- Örtenblad B see Myrbäck K
231
- Oettel H 285
- Ogsten A G and Peters R H
113
- Ogsten A G see Moggridge
R C G 113
- Ohdake S 101 102 198
- Ohle H 588
- Ohle H Erbach H and Carl
H 314
- Ohlmer P 230
- Ohlmeyer P see Meyerhof O
279
- Ohta M see Matukwa T
576
- Ohta T see Takeda Y 42
- Okada S see Suzuki U 220
- Okrent A see Wachholder A
292 319 322
- Okunuki K and Yakusiz E
174
- Olcott H S 437 438 440 462
- Olcott H S and Emerson O
H 440
- Olcott H S and McCamm
D C 54 56
- Olcott H S and Matill H A
437 438 440 454 462
- Oleth H 561
- Oleson J J Burd H R
Elvehjem C A and Hart
E B 214 254 269 477
- Oleson J J see Elvehjem
C A 469
- Oleson J J see Hegsted D M
214 216 474 477 542
- Olefabrik Aarhus and Christen
sen C E 565
- Olefabrik Aarhus and Husein
K. H 570
- Olney M B see Rinehart J F
334
- Olson T M 4 8
- Olsson N 416
- Onstott R H see Sebrell
W H 188
- Opie J W see Evans H M
449 451
- Opie J W see Smith L I
447
- Oppenauer R see Reichstein
T 290 291 296 307 314
317
- Oppenheim E 600
- Oppenheimer C and Stern
K G 175 126
- Opp R L 93
- Orent E R see Litter S
156
- Orla Jensen A D see Dam H
494
- Orla Jensen S Otte N C and
Snog Kjaer A 193 270
- Orla Jensen S see Dam H
494
- Ortoleva G 142
- Osborne T B and Mendel
L B 12 37 154
- Oser B L see Sulzberger
M B 330
- Ost H 274
- Osterberg A F 494
- Osterberg A F see Buit H R
487 504 506
- Osterberg A E see Snell A
M 507
- Osternd T 316
- Ostwald R see Karter P 16
- Ota S and Umeda K 574
- Otake R 576
- Ott E Krämer K and Faust
W 301 315 379
- Ott G L see Coombes A I
55
- Ott M 318 319
- Otte N C see Orla Jensen S
193 270
- Otto H and Rühmkorb F
128
- Ovahoff J see Bruins H R
6
- Owe A W 561 562 564 566
571
- Owen C A see Smith H P
505 507 508
- Owen C A see Ziffern S E
508
- Owens H S Trautman M
and Woods E 269 545
- P
- Pacini A E and Taras M H
80
- Pacini A J 596-598 600 609
- Pacini E and Mullin J W
262
- Page I H 418

- Pagel W see Gundel M 477
 Pagel W see Harris L J 331
 Paget M and Berger R 3.2
 Pal J C and Guha B C 292 301
 Paland J see Butenandt A 407 408
 Paland J see Dimroth K 349 364 407 408
 Paley T J 134
 Palladin O W 309
 Palm T A 342
 Palmer E T see Drummond J C 80
 Palmer L S 39
 Palmer L S see Gulliken T W 432
 Palmieri M L see Cowgill G R 145
 Panizzon L see Hartmann M 585
 Pautschenko-Jurewicz W v and Kraut H 329
 Pappenheimer A M 436
 Pappenheimer A M Goettsch M and Jungherr E 463
 Pappenheimer A M see Goettsch M 436 456 462
 Pappenheimer A M see Hottle G A 475
 Pappenheimer A M see Sherman H C 342 491
 Pappenheimer A M see Zucker T F 343
 Pariente A C see Ralli E P 56
 Park E A 342
 Park E A Guild H G Jackson D and Bord M 336
 Park E A see McCollum E V 343
 Park I O 92
 Parker W F and McFarlane W D 453
 Parks P A see McCollum I V 414 421
 Parott E M see Hogan A G 523
 Parry E G see Barr T 360
 Parson P B see Winegar A H 250
 Parsons H T 476
 Parsons H T see Lease I G 198 199 469 471 473 476-478
 Parsons H T see Strong F M 184 187 192
 Parsons T see Rosenberg H R 595
 Passmore R Peters R A and Sinclair H M 131
 Passmore R see Harris L J 331
 Pasternack R and Brown E V 582
 Pasternack R and Cragwall G O 589
 Pasternack R and Regna P P 307 588 590
 Pasternak S 587
 Patch James A 563
 Patek A J see Haig C 80
 Patrik H see Hogan A G 523
 Patterson C L 555
 Paul W D see Daum A 333
 Paycek P L and Baum H M 275 280 477
 Peacock G see Emmett A D 129
 Pearson P B 249
 Peck F H 558
 Peden O D see Morris A 424 499
 Pederson K O see Kekwick R A 179
 Peebles D D 570
 Pekelharin 10
 Pelczar M J and Porter J R 266
 Pelczar M J see Porter J R 474 479
 Pennington D Snell E E and Fak N R E 476
 Pennington D Snell E F and Williams R J 260 268 270
 Pennington D see Snell E E 266 268
 Pentler C T see Almquist H J 483 494
 Percival E G V see Ault R C 291 308
 Percival E G V see Herbert R W 291 297 299
 Percival E G V see Hirst E I 298 299
 Perino J 596
 Perla D 146
 Perla D and Marmorston J 331 333
 Perlman I and Chaikoff I I 346
 Perlzweig W A Sarett H P and Huff J W 244
 Ferrine T D 578
 Peter V see Busch A H 329
 Peters K 334
 Peters O see Helfferich B 310 588
 Peters R A 170 131 135
 Peters R A and Philpot I St 104
 Peters R A and Rossiter R J 145
 Peters R A and Thompson R H S 148
 Peters R A see Carter C W 529
 Peters R A see Jansen B C P 102
 Peters R A see Kinnersley H W 101-104 120 124 128 130 131
 Peters R A see Long C 136
 Peters R A see McGowan G K 138
 Peters R A see Ochoa S 101 132 139 146 149
 Peters R A see Ogsten A G 113
 Peters R A see Passmore R 131
 Peterson W H McDaniel L E and McCoy E 475
 Peterson W H see Johnson B C 526
 Peterson W H see Lampen J O 286 470 474
 Peterson W H see Snell E E 184 197 193 243 264 268 267 596
 Petrow V A Rosenheim O and Starling W W 413
 Pett L B 89 94
 Pfahler K see Karrer P 164 167
 Pfandler S v 499
 Pfundt R see Reindel F 366
 Phelps F P 58
 Phillips P H and Bohstedt G 36
 Phillips P H and Engel R W 191 269
 Phillips P H see Shaw J H 190 191
 Philpot J S L 176
 Philpot J S L see Angus T C 370
 Philpot J S L see Askew P A 389
 Picher H see Eisenbrand J 583
 Pickel F D see Price D 127 578
 Pickhardt O C and Bernhard A 380
 Pillemmer L see Ecker E E 331
 Pincussen I 476 499
 Pinder J I and Singer J H 500 501
 Pinkerton H and Bessey O A 190
 Pinner A 225
 Pittarelli E 391
 Pittman M and Frazer H F 244
 Pittman M see Daft F S 246
 Placek H 555
 Plass E D see Adler E 174
 Plass M see Adler E 228
 Platt A P 545
 Platt A P see Channon H J 548
 Platt B S and Lu G D 136 148

- Mitz B R see Schneider H
 A 198 211
 Pock Steen P H 19
 Poda ta H H see Wachholder
 k. 3rd
 Pohl R 343
 Poble F J and Stewart J K
 50
 Poble F J see Lozner E I
 3
 Poling C E see György P
 11 71 46 344
 Pollak F 3
 Pollak J E 608
 Polak N L J see Russell W C
 8
 Pons L see Högl F 479
 Poole M W see Slyker F
 40
 Popper H and Greenberg R
 86
 Popper H see Greenberg R
 82
 Popper W see Lepkovsky S
 11
 Porjes N see Wills P A
 304
 Porter C M Porter L V and
 H Block E 557
 Porter J R see Flezar M J
 266 474 40
 Porter L V see Porter C M
 557
 Porter M B see Booher I E
 8
 Portnoy B and Wilkinson J
 F 334 337
 Potter V N R see Lipschutz
 M A 14
 Poucher H G and Stuben
 rauch C H 33
 Powell E L see Hogan A C
 341
 Power F W see Harrow B
 80
 Pratt E F see Weinstock H
 H 28 64 67
 Prebl da H J and McCollum
 E V 13 130 148
 Frewerck E 587
 Prentice J H see Capper N
 S
 Price D and Pickel F D 17
 58
 Price D see Weinstock H H
 265
 Price E A see Carr F H 5
 78 80
 Pricha d W W see E ans
 H V 449 451
 Prehard W W see Smith L
 I 44 447
 Prekett P S see Massengale
 O V 3
 Price R V 554 555
 Prichard H Wilkinson H
 Ed buy J R and Morton
 R A 63 79
- Pulver R and Verzar F 179
 Purr A 328
 Pyke M 100 129 133
- Q
- Quackenbush F W Gottheb
 H L and Steenbock H
 438
 Quackenbush F W and Steen
 bock H 13
 Quarles E see Snell G E 278
 Quastel J H and Webley D
 M 149
 Quensel W and Wachholder
 k. 3rd
 Querido A 414 41
 Querido A Albeaux Fernet
 M and Lwoff A 24J
 Querido A Lwoff A and
 Lataste C 49
 Querido A see Lwoff A 24
 246
 Quibell T H see Karrer F
 181
 Quick A J 50 303 508 508
 Quick A J and Grossman A
 M 307
 Quick A J Stanley Brown
 M and Bancroft F W
 07 308
- R
- Raabe S 329
 Rachele J R see Vignaud V
 du 472
 Raczyński J 342
 Rae J see Griffiths H V
 79
 Raffy A 18
 Raffy A see Guillemond A
 180
 Raffy A see Randon L A
 183
 Ragans J K see Koch F C
 362
 Rahn O and Hegarty C P
 394
 Rahn O Hegarty C P and
 Duclat R E 394
 Rajagopal K R see Moore T
 437 41 406
 Rall E P Frednan G J
 and Kaslow M 320
 Rall E P Pariente A C
 Brandaleone H and David
 son S 30
 Randle S B Sober H A and
 Kohler G O 398
 Randle S B see Kohler G O
 398 329
 Randon L A Raffy A and
 Aguirreabla J 183
 Rane I and Subbarow Y
 250 267 270 544 548
 Rane L see Subbarow Y 260
 266 270
- Raoul Y and Meunier P 79
 Rapoport S 29
 Rathman F H see Lederer E
 71
 Ratsh H D see Scud J V
 391
 Rauch K see Fher F G
 177
 Rauhen H see Wagner Jauregg
 T 174
 Raunert M 17 518
 Raitch I see Hood J S
 410
 Rav S N 319
 Ray S N see Harris I J
 318 323 333
 Rayband I M 560
 Raybin H W 130
 Rymond W D see Harris
 I J 240 244 47
 Reynolds J A 557
 Rea J L and Drummond J
 C 34 36
 Rea J L see Heilbron I M
 60 61 63
 Read C J Strick H C and
 Steck I E 341
 Reader V 591 599
 Reader V see Jansen B C P
 109
 Reader V see Kinnersley
 H W 130
 Reames H R see Dorfman A
 246
 Reckling F 401
 Record P R see Bethke R
 M 418 419
 Reddie J W see McFarlin
 R L 78 5
 Reed C I see Schiller A A
 478
 Redman E J 92
 Reedman E J and McHenry
 F W 299 301 329
 Redman E J Sampson W
 L and Linn A 211
 Reerink F H and Wijk A
 an 344 369-371 41 597
 Reerink F H see Boer A G
 344 346 349 359 369 407
 595
 Reerink F H see Wijk A v n
 380
 Regna P P see Pasternack R
 307 585 590
 Reich H see Gätz Fichter M
 260 266
 Reichel I and Burkart W
 170
 Reich I S v and Deppe M
 393
 Reichel S v see Windaus A
 34
 Reich t in T 991 408 588
 590
 Reichstein T and Demole V
 314

- Reichstein T and Grüssner A
255 280 261 265 301 303
306 307
- Reichstein T Grüssner A
and Oppenauer R 291 290
308 314
- Reichstein T and Oppenauer
R 290 296 317
- Reichstein T Schwarz L and
Grüssner A 314
- Reichstein T see Gätzl Fichter
M 260 266
- Reichstein T see Grüssner A
260 271
- Reichstein T see Hoffer M
264
- Reichstein T see Müller H
314
- Reichstein T see Neracher O
387
- Reid M E 324
- Reilly G see Miles E H
555
- Reindel F and Kipphan H
353
- Reindel P and Niederländer
K 356
- Reindel P Niederländer K
and Pfandt R 366
- Reindel P and Walter E
350
- Reinemund K see Kuhn R
163 166 170
- Reinisch E 561
- Reinke O 559
- Reiter T 601 603
- Reti L 59
- Reverey G see Rosenbusch
R 571
- Rewald B A 565
- Reynolds R J W see Cox
F G 297
- Reynolds R J W see Herbert
R W 291 297 299
- Reynolds R J W see Hirst
E L 208
- Reynolds S 572
- Rhoades C P see Miller
D K 334
- Rhodehamel H W and Klei-
derer E C 597
- Rice E E see Rose W C
540
- Rich A R and Hamilton J
D 544
- Richards G V see Unna K
269
- Richards M B see Irving J
T 93 96
- Richards O W 257
- Richards W F see Ungnade
O 569
- Richardson L R and Hogan
A G 197 286
- Richardson L R see Hogan
A G 523
- Richardson L R see Robbins
W J 126 133
- Richter C P and Hawkes
C D 213
- Richter H see Geflick H
602
- Richtert D Thayer S A
McKee R W Binkley S B
and Doisy E A 494 499
- Rider T H Sperts G Goode
G P and Cassidy H G
369
- Ridgeway R R see Cuthbert
son W F T 437 451 457
458
- Ridout J H see Best C H
543-546
- Ridout J H see MacLean
D L 545
- Ried O 601 603
- Riegel B Schweitzer C E and
Smith P G 483 484
- Riegel B see Fieser L F
406
- Rigler L G see McQuarrie I
372
- Rigo L see Mosonyi J 328
- Rising B M see Baumann
C A 55
- Rinehart J F 334
- Rinehart J F Greenberg L D
Olney M B and Choy
P 334
- Ringier B H see Demole V
449
- Ringier B H see Karrer P
179 233 238 437 442 444
445 449 450 453
- Ringrose A T Norris L C
and Heuser G F 254 269
478
- Ringrose A T see Norris
L C 185 20 246 254 269
- Ringstead A 462
- Ringstead A see Einarson L
462 464
- Ritsert K 128 147 249 413
570
- Ritsert K see Zima O 143
- Ritsert V 80
- Rittenberg D 366
- Rittenberg D see Clutton R
F 541
- Ritzmann J see Goettsch M
462
- Ritzmann J R see Winter-
steiner O 360
- Rivers A B and Carlson L A
334
- Rivers T M 799
- Rivkin H see Hess A F
410
- Robbins R C see Todhunter
E N 335 516
- Robbins W J 132
- Robbins W J and Bartley
M A 133
- Robbins W J Bartley M A
Hogan A G and Richard-
son L R 116 133
- Robbins W J and Kavanagh
F 197
- Robbins W J and Schmidt
M B 212
- Roberts E G see Anderson
R J 276
- Robertson E B 316
- Robertson E I see Carver J
S 420
- Robeson C D see Baxter
J G 60 61 70 81
- Robeznieks I 513
- Robinson E 515
- Robinson H D see Young
F H 563
- Robinson H E Gray R E
Chesley F F and Crandall
L A 284
- Robinson H E and Newton
R C 204 269 477
- Robinson H J see Molitor H
509
- Robinson W D see Melnick
D 241 248 249
- Robinson W L see Bobstedt
G 431
- Robison R 493
- Robison R and Soames K M
423
- Rocholl H see Lauber H J
84
- Roe J H 321
- Roe J H and Barnum G L
327
- Roe J H and Hall J M
336
- Roe J H see Kassar R J
335
- Roedig A see Fisher F G 177
- Roethm R R see Williams
R J 134 266
- Rogers G D 561
- Rogers L M see Goldberger
J 220 242
- Rogers R E see Stokstad
E L R 525 517 513
- Rohrmann C see Williams
R J 255 257 266 267
- Rolf I P see Leene P A
572
- Roller D see Lasch F B
- Roller R 92
- Rollett A 534
- Rominger E 423 494 499
- Rominger E Meyer H and
Bomskov C 429
- Rosen Runge C see Windaus
A 393 400
- Rosanova V see Gillam A E
68
- Rosanova V see Lederer E
68
- Roscoe M H see Aykroyd
W R 220
- Roscoe M H see Chalk H
107 137 156 197
- Rose C S see Brown H B
414 421

- Rose C S see György P 474
 478
 Rose E and Sunderman T W
 380
 Rose W C 540 541 548 549
 Rose W C and Rice E E
 540
 Roewer W C see Scull C W
 441
 Rosedale J L 102
 Rosen C see Evelyn A A
 319
 Rosenberg A see Geiger A
 142
 Rosenburg H R 347 361 406
 595
 Rosenberg H R and Parsons
 T 595
 Rosenberg H R and Tinker
 J M 360 595
 Rosenburg H R see Ruzicka
 L 20 363
 Roenberger F 776
 Rosenblum L A and Jolliffe
 N 248
 Rosenblum L A see Jolliffe
 N 248
 Rosenbusch R and Revery
 C 571
 Rosenheim O 367
 Rosenheim O and Drummond
 J C 80
 Rosenheim O and Webster
 T A 80 343 371
 Rothenberg O see Petrow
 V A 413
 Rosenstein R v 8
 Rosenthal J and Erdélyi J
 79 80
 Rosenthaler L 32
 Rossiter R J see Orboa S
 184 188 192
 Rosier R J see Peters R A
 145
 Rost H F 601
 Rothschild E see Dam H
 48 505
 Rotter H 337
 Ropppe 8
 Rowan W 406
 Rowlands I W and Singer E
 459
 Rowley C D see From V C
 601
 Roy G 611
 Roy G K see Wilson H E C
 211 214
 Rubbo S D and Gillespie J
 M 253-286
 Ruben S and Kamen M D
 137
 Rubinstein D and Shekun L
 42 250
 Rudolph H see Braun J v
 65
 Rudra M N 313 330 333
 Rudy H 40 124 179 439
 Rudy H see Kuhn R. 107-
 159 163 172 177 179 184
 58 583
 Ruegger A see Karrer P
 66 69 70 76 483
 Ruehle A E see Cline J K
 100 109
 Ruehle A F see Williams R.
 R 104 105 109 113 114
 Ruehl A E see Wintersteiner
 O 103
 Rühmekorb F see Otto H
 1 8
 Ruhkopf H see Michael F
 278
 Ruhkopf H see Wandaus A
 100 107
 Runnström J and Michaelis
 L 237-236
 Rupel I W Bohsted G and
 Hart I B 437
 Rupel J W see Bumann C
 A 86
 Ruskin S L 585 592
 Russell G R see Gerstenberger
 H J 42
 Russell W C Taylor M W
 Walker H A and Polson
 L J 85
 Russell W C see Nield C H
 413
 Rusznayk L and Benko A
 516
 Rusznayk I and Szent
 György A 513
 Rusznayk I see Armentano L
 513 517 518
 Rusznayk I see Benisz A
 513 516
 Ruzicka L 43 359 571
 Ruzicka L and Fischer W
 76
 Ruzicka L Goldberg M W
 and Rosenberg H R 20
 Ruzicka L and Rosenber
 H R 363
 Rydbom M see Eiler H v
 75
 Rydbom M see Karrer P 76
 Ryden J O see Clausen S W
 89
 Rygh O see Wandaus A 363
 408 418
 Rzeppa H W see Kuhn R
 181
 S
 Sab P P T 305 36 491
 Sab P P T and Brüll W
 491
 Sab P P T Brüll W and
 Holzen H 491
 Saha K C 92 301
 St John J L see Carver J S
 420
 St Philpot I see Peters R A
 104
 Salkowski E and Salkowski
 H 2.6
 Salkowski H see Salkowski
 E 26
 Salmon W D 213
 Salmon W D Guerrant N B
 and Hays I M 107 155
 Salmon W D see Engel R W
 545
 Salomon A 597
 Salomon H see Dam H 482
 505
 Salomon H see Demole V 449
 Salomon H see Karrer P
 46 65 106 100 163-165
 167 181 296 298 437 442
 444 445 449 450 453 457
 483
 Salzberg P L 582
 Salz W 584
 Samant K M see Heilbron
 I M 376
 Sampson W L see Fieser L
 F 487 495-497 499 500
 Sampson W L see Molitor
 H 104
 Sampson W L see Reedman
 E J 211
 Sampson W L see Tishler
 M 496 500
 Sampson W L see Unna K
 269
 Sanchez Rodriguez J and
 Sarda J M 128 145
 Sand r C E see Brinch U
 558
 Sandulesco G and Girard A
 561
 Sanford T D see Jakes T H
 384 410
 Sarda J M see Sanchez
 Rodriguez J 18 143
 Sarett H P see Perlzweig
 W A 244
 Sartory A Sartory R and
 Meyer J 324
 Sartory R see Sartory A 324
 Sassaman H L see Bethke
 R. M 418
 Sato M see Ixume S 575
 Sotda I see Kematsumi S
 25
 Satterfield G H see Yokota
 A D 3 6
 Sauer E M see Brown
 H B 414 41
 Saunders D H see Williams
 R J 257-266
 Saunders F Dorfman A and
 Koser S A 248
 Saunders F see Dorfman A
 246 249
 Saunders F see Koser S A
 243
 Scanlon G H Brinkhaus
 K. M Warner E D Smith
 H P and Flynn J E
 506 50

- Scarborough H 513 514 518
 Scarborough H and Stewart
 C P 302 301 317 320 329
 333
 Schabad J A 4 3
 Schardinger F 176
 Schaumann H 521
 Scheer B T 87
 Schenck F 344
 Schenck F see Windaus A
 344 360 595 604
 Schenck R T see Engels
 U H 591
 Scheunert A 30 91
 Scheunert A and Schieblisch
 M 74 313
 Scheunert A and Wagner K
 H 42 43
 Scheunert A see Kuhn R
 74
 Schieblisch M see Kuhn R
 74
 Schieblisch M see Scheunert
 A 74 313
 Schiedt B see Maurer K
 314
 Schiff E 60
 Schiff E and Hirschberger C
 625
 Schiller A A Struck H C
 and Reed C I 428
 Schindl L see Heintschel H
 405
 Schindl L see Schönheimer
 R 366
 Schindler A 607
 Schiro H S see Vilter R W
 215
 Schlemmer F Bleyer B and
 Cahmann H 295 320
 Schlenk F 232 235 237
 Schlenk F and Euler H v
 232
 Schlenk F Hellström H and
 Euler H v 229
 Schlenk F see Albers H 232
 586
 Schlenk F see Euler H v
 220-223 230 232 236 240
 241 244 250 522 585
 Schlienz W see Karrer P
 38 41
 Schlatter E see Karrer P
 160 163 164 181
 Schlötzer A see Euler H v
 183
 Schlubach H and Vorwerk J
 304
 Schlutz F W and Morse M
 343
 Schmel M see John W 447
 450
 Schmelkes F C 127 240
 Schmelkes F C and Joiner
 R R 127
 Schmidt A A and Tutschins
 kaja K S 588
 Schmidt C L A 504 506
 Schmidt C L A see Greaves
 J D 56 84 91 418 456 504
 Schmidt C L A see Tarver
 H 549
 Schmidt G see Euler H v
 68
 Schmidt H see Winegar A H
 250
 Schmidt J 557
 Schmidt M B see Robbins
 W J 212
 Schmiere E 556
 Schmitt C 555
 Schnabel C F 555 556
 Schnabel C F see Smith L H
 557
 Schneider H see Sperts G
 598
 Schneider H A Ascham J K
 Platz B R and Steenbock
 H 198 211
 Schoch 421
 Schoenebeck O von see Klenk
 E 532
 Schoenenberger W see Buhr
 T 570
 Schönheimer R 359
 Schönheimer R Behring H v
 Hummel R and Schindl L
 366
 Schönheimer R see Clutton
 R F 541
 Schöpp K see Euler H v 167
 181
 Schöpp K see Karrer P 53
 54 60 80 156 160 163-165
 167 181 296-298
 Schörmüller J 155
 Schöneyder F 482 506
 Schöneyder F see Dam H
 481-483 506 507 509
 Schoor A van 198
 Schopfer W H 131 134
 Schopfer W H and Blumer S
 455
 Schopfer W H and Jung A
 131 134
 Schotland C E see Antopol
 W 214
 Schour L Smith M C and
 Hoffman M M 93
 Schürfler C B see Dimick
 M K 211 214 216
 Schreiber B see Gams A
 573
 Schroeder H 147
 Schroeder H see Stepp W
 193 212
 Schroeder H see Wendt H
 91
 Schuck W P 566
 Schuette H A 8
 Schuette H A see Woessner
 W W 317 318
 Schubknecht W 412
 Schultz A S Atkin L and
 Frey C N 131 134 148 211
 212
 Schultz F 478 609 610
 Schultz F see Windaus A
 100 102
 Schultz H W see Boussevain
 C H 185
 Schultz O K see Kersten G
 602
 Schultze H E 606
 Schultze M O see Stotz E
 377
 Schulze G E R 356
 Schumacher A E and Heuser
 G F 191
 Schumacher A E see Bauern
 fand J C 253
 Schuster P see Lohmann K
 100 123 124 126 136 146
 580
 Schuyt J W and Groen J
 190
 Schuyt J W see Groen J
 192
 Schwab G see Karrer P
 450
 Schwartz C see Hamilton B
 409 475
 Schwartz K 316
 Schwartz W P see Ecker E
 B 331
 Schwarz L see Reichstein T
 314
 Schwarzenbach G see Karrer
 P 238 296 297 298
 Schwarzenbach G see
 Michaelis L 162
 Schweitzer C E see Fieser L
 F 496
 Schweitzer C E see Riegel B
 483 484
 Scott H M Hughes J S and
 Loy H W 410
 Scott W 578
 Seudi J V 500 584
 Seudi J V Koonen H F and
 Keresztesy J C 199 210
 212 215
 Seudi J V and Ratish H D
 321
 Seudi J V Unna K and
 Antopol W 210 212
 Seull C W and Rose W C
 541
 Sealock R R and Silberstein
 H E 330
 Searcy 220
 Sebrill W H 242 247
 Sebrill W H and Butler R E
 191
 Sebrill W H Butler R E
 Woolley J G and Isbell H
 193
 Sebrill W H and Onstott R
 H 188
 Sebrill W H Wheeler G A
 and Hunt D J 242
 Sebrill W H see Daft F S
 246 269 271 544

- Sebrell W H see Frazer H F 242
 Sebr H W H see Goldberger J 270
 Sebrell W H see Kruse H D 191
 Sebrell W H see Lillie R D 544
 Sebrell W H see Oden J W 191
 Seel H see Brauer L 605
 Seibert H F 580
 Seiberth M see Hartmann M 585
 Seidell A 10 155 156 573 575
 Seidell A and Smith M I 102
 Seps J 603
 Setz F 369
 Selbie F R 283 285 286
 Seifridge G 247
 Sell M T see Steenbock H 53
 Sen Gupta P N and Guha B C 292 301 319
 Sen Gupta P N see Guha B C 292 301 319 326
 Seto I see Iume S 575
 Setz P 372 381
 Seyffert E 224
 Shaler S see Hecht S 89 93
 Sharp P F see Hand D B 292
 Shaw G E and MacDonald C A 240
 Shaw J H and Phillips P H 190 191
 Shear M J 413
 Sheets O and Funk C 343
 Sheets R F and Struck H C 91
 Shekun L see Rubenstein D 242 250
 Shepherd M I and Lann D R 537
 Sherman H C 153 546
 Sherman H C. and Axtmayer J H 99 156
 Sherman H C. and Lanford C S 186
 Sherman H C. and Pappenheimer A M 342 421
 Sherman H C and Smith S L 7 131 132
 Sherman H C and Spohn A 131 132
 Sherman H C see Bourquin A 183 254 269 477
 Sherman H C see Carlsson B V 183 192
 Sherman W C see Elvehjem C A 101
 Sherwin C P see Harrow B 283
 Sherwood C R see Drill V A 145
 Sherwood R C and Ferrari C G 606
 Sherwood R M and Fraps G S 96
 Shettles L B Dells E and Hellman L M 505
 Shuls M Day H G and McCollum E V 148
 Shumamura T see Suzuki S T 99 220
 Shimizu F 570
 Shimotori N Emerson G A and Evans H M 462
 Shinowara G Y and Brown J B 533-535
 Shibley P G 422
 Shipley P G see McCollum E V 343 414 421
 Shohl A T 409 475
 Shohl A T and Brown H B 425
 Short D M see Crimm P D 96
 Shrewsbury C L see Hogan A G 573
 Shukers C F see Langston W C 524
 Shumway E D 598
 Shute E V 463
 Sidwell A R see Hogness T R 350
 Siebert F 319
 Siegel J 605
 Summers G F 557
 Seve B F 284 286
 Sigal A and King C G 330-333
 Silberstein H E see Sealock R R 330
 Silverman M and Werkman C H 125
 Simmonds N see McCollum E V 342 343 414 421
 Simms H D see Morgan A F 253
 Simola P E 30 332
 Simons E J H and Zucker T F 344 387 388
 Simons E J H see Buxton L O 554 565
 Simpkins G W see Edisbury J R 58 68 71
 Simpson G C E see Ed e C S 99
 Simpson J C E see Heilbron I M 354
 Simpson J W and Mason K E 92
 Sinclair H M 131 134 149
 Sinclair H M see Goodhart R S 135 149
 Sinclair H M see Passmore R 131
 Sinclair R D 431
 Sinclair R G 538
 Sing I S A see Sydenstricker V P 477
 Singer E 460
 Snger E see Drummond J C 438 440
 Snger E see Rowlands I W 439
 Snger J H see Pinder J L 500 501
 Suzoo G J see Dolz M J L 473
 Sjögren B 496
 Sjölema B 478 477
 Skelley W C 431
 Skraup Z H 273
 Skraup Z H and Cobenzl A 224
 Skraup Z H and Vortmann G 274
 Sloan L L see Derby G S 93
 Sloan R 335
 Slotin L see Evans F A 137
 Slyker F Hamel B M Poole M W Cooley T B and Macy I G 420
 Smakula A 104 105 370 371 390
 Small J M D see Morris N 424
 Smedley MacLean I and Hofert D 365
 Smedley MacLean I and Hume E M 538
 Smedley MacLean I and Nunn L C A 538
 Smedley MacLean I see Hume E M 538 537 539
 Smedley MacLean I see Nunn L C A 531 535 538
 Smirnov A P 279
 Smith A H and Meyer C E 138
 Smith A M and Gilles J 324
 Smith A R 556
 Smith F L and Hazley V 79
 Smith E L Stern B E and Young F E 39 65 71 81
 Smith E L see Bacharach A L 59
 Smith F see Ault R G 791 308
 Smith F see Baird D F 291 308
 Smith F see Haworth W N 588
 Smith F see Herbert R W 291 297 299
 Smith F see Hirst E L 298 299
 Smith G G R 568
 Smith H H see Hume E M 417 436 537 539
 Smith H P and Owen C A 505
 Smith H P Warner R D and Brinkhaus K M 603

- Smith H P Ziffert S E
Owen C A and Hoffman
G R 508
- Smith H P Ziffert S E
Owen C A Hoffman G R
and Flynn J E 507
- Smith H P see Brinkhaus
K M 507
- Smith H P see Scanlon G H
508 507
- Smith H P see Tidrick R T
502
- Smith, H I see Warner E D
482 505 507 508
- Smith H P see Ziffert S E
508
- Smith J A B see Channon
H J 546 —
- Smith J H C 75
- Smith L H and Schnabel
C F 557
- Smith L I Irvin W B and
Ungnade H E 453
- Smith L I and Ungnade H
E 607
- Smith L I Ungnade H E
Opie J W Prichard W W
Carlin R B and Kaiser
E W 447
- Smith L I Ungnade H E
and Prichard W W 445
- Smith L I and Webster I M
491
- Smith L I see Emerson O H
430 444
- Smith L I see Evans H M
440 451
- Smith L I see Ungnade H E
454
- Smith M C and Spector H
418
- Smith M C see Schour L 93
- Smith M I 130 132
- Smith M I and Hendrick E
G 150
- Smith M I see Seidell A
102
- Smith P G see Fieser L F
496
- Smith P G see Riegel B 483
484
- Smith S G see Margolis G
247
- Smith S G see Margolis L H
192
- Smith S L 337
- Smith S L see Sherman H C
7 131 132
- Smyth D H see Krebs H A
137
- Snell A M 507
- Snell A M Magath T B
Boland E W Osterberg
A E Butt H R Bollman
J L and Walters W 507
- Snell A M see Butt H R
481 482 491 504 506 508
509
- Snell A M see Clark R L
501 507
- Snell E E Eakin R J and
Williams R J 474 475
- Snell E C Pennington D and
Williams R J 266 268
- Snell E C and Peterson W
H 526
- Snell E F and Quarles E
278
- Snell E E and Strong F M
184 185 192 193
- Snell E E Strong F M and
Peterson W H 184 192
193 243 264 266 267
- Snell E E and Williams R J
475
- Snell E E and Wright L D
242 266 474
- Snell E E see Eakin R E
476
- Snell P F see Frauskopf
F J 185 267 270
- Snell E E see Mitchell H A
256 258 263-265 525
- Snell, E E see Pennington D
266 268 270 476
- Snell E E see Stanbery S R
268 270
- Snell E C see Williams R J
260
- Snelling W O 503
- Snadecki J 342
- Snyder R H and Bloor W R
538
- Snog Kjær A see Orla Jensen
S 193 270
- Snow C P see Bowden F P
81 995
- Snow G A and Zylva S S
322
- Soames K M see Goldblatt
H 418
- Soames K M see Robison R
423
- Sobel A C see Kramer B
599
- Sober H A Lipton M A and
Elvehjem C A 138
- Sober H A see Axelrod A E
192
- Sober H A see Randle S B
576
- Sobotka H see Willstätter R
554
- Sobotka M Halden W and
Bilger F 366
- Soder H see Euler H v 523
- Sohl A T and Wolfbach S B
414 421
- Sohl A T see Brown H B
414 421
- Solmsen U V see Euler H v
70 76
- Solmsen U V see Foster
R H K 494 499
- Solmsen U V see Karrer P
76 233 238
- Solomon A K Vennesland B
Klemperer F W Buchanan
J M and Hastings A B
542
- Solomon A K see Conant J
B 542
- Sonderhoff R and Thomas H
365
- Sophie W 247
- Southern S P see Flot M M
427
- Spadola J M and Ellis N R
538
- Sparth C P see Lawson W
F 582
- Spector H see Smith M C
418
- Spehr H see Brunch U 558
- Spellberg M A and Keeton
R W 544
- Spencer H C see Morgulis S
458
- Sperli G 598
- Sperli G Norris R J With
row R B and Schneider H
598
- Sperli G see Rider T H
369
- Spiegelberg H 578
- Spies T D Bean W B and
Ashe W F 214 216
- Spies T D Bean W B and
Stone R E 245 246
- Spies T D Bean W B Vilter
R W and Huff N E 191
- Spies T D Hightower D P
and Hubbard L H 214 215
249 270 464 523
- Spies T D Ladisch R K and
Bean W B 215
- Spies T D Stanbery S R
Williams R J Jukes T H
and Babcock S H 268
- Spies T D and Vilter R W
464
- Spies T D Vilter R W and
Ashe W F 192 249
- Spies T D Walker A A and
Woods A W 240 249
- Spies T D see Axelrod A E
188 192 244
- Spies T D see Bills C E
240
- Spies T D see Blankenhorn
M A 240
- Spies T D see Stanbery S R
268 270
- Spies T D see Vilter S P
192 214 215 233 234 239
241 243 244 247 248
- Spies T D see Williams R R
99 113
- Spindle F see McGinnes G F
575
- Spohn A see Sherman H C
131 132
- Spring F S see Barr T
360

- Spring F S see Hellbron I
M 367 368 376 396 399
413
- Spring F S see Kennedy T
390
- Spruyt J P 130
- Sreenivas ya M see Adler E
2 8
- Stacey M see Ault R G 91
308
- Stacey M see Baird D A
291 308
- Stanbery S R Snell E E and
Spes T D 268 270
- Stanbery S R see Mitchell
H K 256 258 265
- Stanbery S R see Spes T D
268
- Stanley Brown M see Quirk
A J 507 508
- Stantial H 278
- Stare F J 156
- Starling W W see Petrow
V A 413
- Starzynski B 316
- Stratus G Jeans P C and
Vandecar V 420
- Stearns G see Jeans P C
430
- Steck I E see Read C I
341
- Steenbock H 37 53 343 596-
598
- Steenbock H and Black A
343 404 414 415 421
- Steenbock H and Boitwell
P W 53
- Steenbock H and Gross E G
53
- Steenbock H Hart E B
Elvehjem C A and Kletsien
S W F 385
- Steenbock H., and Nelson M
T 343
- Steenbock H Sell M T Nel-
son E M and Buell M V
53
- Steenbock H see Baumann
C A 55 86
- Steenbock H see Hamann R
W 362 368
- Steenbock H see Kon S K
362 368 409
- Steenbock H see Quackenbush
F W 213 438
- Steenbock H see Schneider
H A 198 221
- Steenbock H see Waddell J
452
- Steiger M see Jacob A
446
- Steigmann A 413
- Stein G A 579
- Stein G A see Stevens J R
578
- Stein G A see Wundus A
381
- Steinberg C L 464
- Steinhaus A H see Levine J
404
- Stephens D J and Hawley
E F 326
- Stephens D J see Hawley
E E 325 326
- Stephens H C 556
- Stephens H C and Hoar
S B 599
- Stepp W 10 37 124 439
- Stepp W Kuhn u J and
Schroeder H 193 12
- Stern B E see Smith H L
39 65 71 81
- Stern K G and Hofer J W
125
- Stern K G and Melnick J L
144
- Stern K G see Oppenheimer
C 125 126
- Stern R O see Findlay C M
477
- Stern R O see Zeller E A
177
- Stevens D L see Hawley E
E 336
- Stevens J R and Stein G A
578
- Stevens J R see Keresztesy
J C 198 199 211
- Stevens J R see Stiller E T
200 202
- Stevenson M M see Morris
N 412 424
- Stewart C P see Scarborough
H 292 301 317 325 329
333
- Stewart J K. see Pohle F J
506
- Stewart P A see Hellbron
I M 376 399
- Still E W see Kern R
380
- Still E T Harris S A
Finkelstein J Keresztesy
J C and Folkers K 255
257 61 264 271
- Still E T Keresztesy J C
and Finkelstein J 209
- Still E T Keresztesy J C
and Stevens J R 200-20
- Still E T and Wiley P F
260
- Still E T see Harris S A
204
- Still E T see Keresztesy
J C 198
- Stiner O Hauswirth A and
Gams A 554
- Sturmman E see Groll C
593
- Stockard C R 431
- Stocker H see Weygand F
172
- Stockholm M Althausen T
L and Borson H J 134
- Stoeltzner W 413
- Stoerr E see Berrsonoff N
336
- Stoesser A V McQuarrie I
and Anderson J A 544
- Stoesser A V see McQuarrie
I 379
- Stokstad E L R 6
- Stokstad E L R Almquist
H J Mecchi F Manning
P D V and Rogers R E
513
- Stokstad E L R and Mann-
ing P D V 191 5 5
- Stokstad E L R Manning
P D V and Rogers R E
525 542
- Stokstad E L R see Almquist
H J 483 484 505 542
- Stoll A see Willstätter R 44
51 52
- Stoll W see Ahrens G 375
- Stone I 539
- Stone I see Gray P P 591
- Stone R E see Spies T D
245 246
- Stone S 464
- Stoner G C see Brown J B
534
- Storvick C A see Belser W
B 335
- Stots E Harrer C J Schultze
M O and King C G 327
- Str in H H 44
- Straub F B 173 175
- Straub F B Corran H S
and Green D E 173
- Straub F B see Corran H S
173 174
- Straub F B see Laki K
138
- Strauss E Lowell F C and
Finland M 286
- Street H R and Cowgill
G R 188 191
- Ströbele R see Kuhn R 161
166 167 170 582
- Ström J 431
- Strohecker R and Vaubel R
318
- Strong F M Feeney R E
Moore B and Parsons H T
184 187 192
- Strong F M see Elvehjem
C A 220 242 250
- Strong F M see Feeney R E
184 19
- Strong F M see Karrer P
167
- Strong F M see Snell E F
184 185 193 243 264
266 267
- Strong F M see Woolley
D W 39
- Strick H C see Red C I
341
- Struck H C see Schuller A A
428

- Struck H C see Sheets R F 91
 Stuart E H 575 591
 Stuart E H see Sure B 575
 Stubbs J J see Wells P A 304
 Stubenrauch C H see Poucher H G 337
 Stumpf P K see Green D E 171
 Subbarow Y and Hitchings G H 256 270
 Subbarow Y and Rane L 265 267 270
 Subbarow Y see György P 269
 Subbarow Y see Hitchings G H 266 269
 Subbarow Y see Rane L 260 267 270 544 548
 Subbarow Y see Trager W 103
 Subrahmanyan V Green D F and Gordon A H 176
 Subrahmanyan V see Gordon A H 176
 Subrahmanyan V see Green D E 123
 Suckfull F see Michael F 2 8
 Söltmann H and Burkhäuser H 142 143
 Sullivan M X 245
 Sultzberger M B and Oser B L 330
 Sulzmann F W see Rose E 380
 Supplee G C 597 598 606
 Supplee G C Ansbacher S Bender R C and Flanigan G E 409
 Supplee G C Ansbacher S Flanigan G E and Hanford Z M 182
 Supplee G C Bender R C and Jensen O G 183
 Supplee G C and Dorcas J 599
 Supplee G C and Flanigan G E 573
 Supplee G C Flanigan G E and Bender R C 556
 Supplee G C see Ansbacher S 156 580
 Supplee G C see Bender R C 211
 Supplee G C see Flanigan G F 581
 Sure B 283 286 435 5 4 545 575
 Sure B and Buchanan K S 140
 Sure B Kik M C and Buchanan K S 89
 Sure B and Stuart E H 575
 Sure B Theis R M and Harrelson R T 332
 Sure B see Jones W A 146
 Sutchiff F K see Jacob A 449
 Suzuki U 220
 Suzuki U and Matsunaga S 220
 Suzuki U Shammura T and Okade S 99 220
 Svensson T see Kuler H v 399
 Svrbely J L 332
 Svrbely J L and Szent György A 291 293
 Swaminathan M 240 249
 Swanson L W see Greene J A 380
 Swingle K F see Dorfman A 246
 Swonalaenen P 98
 Sydenstricker V P Singal S A Briggs A P DeVaugh M N and Isbell H 477
 Sydenstricker V P see Kruse H D 191
 Szent György A 173 289 291 327 328 513 514 516 517 610
 Szent György A and Haworth W N 289
 Szent György A see Armen tanó L 513
 Szent György A see Banga I 154 177
 Szent György A see Bentsáth A 513 516
 Szent György A see Bruckner V 514
 Szent György A see Laki K 138
 Szent György A see Rus zynák I 513
 Szent György A see Svrbely J L 291 293
 Szatarczy G v 313
- T
- Taffel M and Harvey S C 333
 Tage Hansen E see Dam H 482 506 507 509
 Taguchi K 575
 Takahashi K 57 562
 Takaki K 8 100
 Takamine J Takamine J and Fujita N 554
 Takeda Y and Ohta T 42
 Takeda Y see Grundmann C 42
 Tanner W F see Goldberger J 219
 Tanret C 343 346
 Tansley K see Lythgoe R J 90
 Taras M H see Pacini A E 80
 Tarver H and Schmidt C L A 549
 Taube L see Lange F 573
 Tauber H 124 130 135 321 328 580
 Tauber H and Kleiner I S 322 335
 Tauber H and Laufer S 284
 Tauber H see Weiglard J 125
 Taylor F Chase D and Faulkner J 335
 Taylor F H L see Faulkner J M 325 335
 Taylor F H L see Lorzer E L 325
 Taylor G F and Day C D M 478
 Taylor H F 557 565 572
 Taylor H F Wells A W and Nedvedsky V A 564
 Taylor L W see Lepko sky S 188 191
 Taylor M W see Russell W C 85
 Taylor T C and Nelson J M 532
 Tchowski C T see Martin G J 285
 Tchowski C T see Wi ansky W A 285 286
 Tecklenburg M L see Brock mann H 55 78 79
 Tecklenburg M L see Kuhn R 73
 Test W H 570
 Tew W P see Watson E M 463
 Thayer S A Binkley S B MacCorquodale D W Doisy E A Emmett A D Brown R A and Bird O D 603
 Thayer S A MacCorquodale D W McKee R W and Doisy E A 504
 Thayer S A McKee R W Binkley S B MacCorquodale D W and Doisy E A 502
 Thayer S A see Binkley S B 487 484-490 493 499
 Thayer S A see MacCorquodale D W 487 493
 Thayer S A see McKee R W 482 484 486 489 496
 Thayer S A see Richtert D 494 499
 Theis R M see Sure B 332
 Theorell H T 172-174 593
 Thernault E J 202
 Thiele W and Trautmann G 395
 Thiele W see Windaus A 393

- Th enes C H see Leser A J 517
 Thom s B H see Eck J C 409
 Thomas B H see Yoier L 409
 Thomas H see Sonderhoff R 365
 Thompson 543
 Thompson H E see Beard H H 369
 Thompson H W see Castle D C 63
 Thompson M R see Martin G J 245 79
 Thompson R H S and John son R E 136 149
 Thompson R H S see Peters R A 148
 Thompson S Y see Houston J 101 147
 Thomp on W H see Mc Q arre I 379
 Thornton J J see Cerecedo L R 118
 Thurman B H 564
 Tidrick R T Joyce F T and Smith H P 502
 Till ch H C I 561 600
 Tillmans H and Hirsch P 30
 Tillmans J 291 316
 Tillmans J., and Hirsch P 91
 T n S see Yamamoto R 41
 Tinker J M see Rosenberg H R 360 395
 Taker O A see Baxter J G 61
 Tischer A O 59 563
 Tischer A O see Hickman K C D 567 568
 Tisdall F F 601
 Tisdall F F see Drake T G H 410
 Tisdall F F see Kramer B 49
 Tisher J 42
 T hler M Fieser L F and Sampson W L 500
 T hler M Fieser L F and Wendler N L 450 493 496 497 499 500
 Tishler M and Sampson W L 496
 Tishler M and Wellman J W 163 531
 Tshler M see Fieser L F 482 490-497 499 500
 Titus H W see DeVaney G M 406 419
 Todd A R and Bergel F 116 18
 T dd A R Bergel F Frae kel Con t H L and Vetter H 10
 Todd A R Bergel F and Jacob A. 111
 Todd A R Bergel F and Karimullah 111
 Todd A R Be gel F Kar m llah and Keller R 111
 Todd A R Bergel F and Work T S 437 438 448
 Todd A R see Barger G 111 120 126-1-8
 Todd A R see Bergel F 441 443 440
 Todd A R. see El Sadr M M 264
 Todd A R see Jacob A 446 449
 Todh nter E N 35
 Todhunter E N and Robbins R C 335
 Todhunter E N Robbins R C Ivey G and Brewer W 516
 To nis B see K6gl F 471 474
 Topelmann H nd Schuh knecht W 412
 Tolle C D see Kline O L 130
 Toml nson M L 110
 Topping M C and Knoefel A F 192
 Topping N H see Frazer H F 188 192 249
 Tornya E 594
 Tor6 E see Jeney A v 330
 Torrance C C 331
 Tortell M and Jaff6 F 413
 Totter J R and Day P L 541
 Tourtellotte D and Bacon W E 417
 Tovernd C see Tovernd K W 419 430
 Tovernd K W and Tovernd G 419 430
 Town end E T and Mills E S 507
 Toyama Y and Tsuchiya T 535
 Trager W and Subbarow Y 193
 Trautman M see Owens H S 269 545
 Trautmann G see Thiele W 395
 Trautmann G see W ndaus A 390 400
 Trav s P M and Glaban C 537
 Tr6fouel J Tr6fou I J Nitt F and Bovet D 90
 Trenner N R and Bacher F A 500
 Tribout A 603
 Triggs W W 539 574
 Tripp F see Holmes A D 36
 Tratram G R see Channon H J 548
 Trouseau A 34
 Truesdail T H see Williams R J 903 755 57 760
 Tschesche R see Wind us A. 100 102 100 109
 Tschopp E see Mescher K 20
 Tschugajeff L 368
 Tso E see Hou H C 406
 Tsuch ya T see Toyam Y 530
 Tsuzuki J 573
 Tucker H F and Eckste n H 543
 Tufs E V and Greenberg D M 190
 Tully R H see Longenecker H E 313
 Tully R H see Mitsul n R R 312
 Turpe nen O 531 537
 Tutsch nskaja K S see Schmidt A A 538
 Tutt J P 463
 Tunson P see Zechmeister L 38
 Twyman F and Follet D H 572
 Tzon H 41
 Tzoni H see Halden W 412
- U
- Uber F M and Verbrugge F 104
 Ugam S s e Nakahara W 54
 Um da k. see Ota S 574
 Underbjerg G k L 465
 Underhill F P see Ch tt nden R H 0
 Underhill S W F and Coward K H 54 75
 Underhill S W I see Mead T H 81
 Underkofler L A see L sh J B 978
 Unger F 566
 Unger L J see He s A F 99 295 343
 Ungley C C see Harris L J 147
 Ungnade H F and Smith L I 454
 Ungnade H E see Evans H M 449 451
 Ungnade H E see Sm th L I 445 447 453 607
 Ungnade O 39
 Ungnade O and Richard W F 569
 Unna k. 909 215 969
 Unna k. and Antopol W 215
 Unn K and Gre lin J 270
 Unna k. Richards G v and Sampson W L 269 1
 Unna K see Antopol W 214

- Unna K. see Reedman E J 211
 Unna K. see Scudi J V 210 212
 Urbain G. see Mentzer C 312
 Urbaneck J 334
 Utzinger G F. see Karrer P 238
- V
- Vandecar V. see Stearns G 420
 Van de Heuvel F A. see Farmer E H 336
 Vandembelt J M. see Ewing D T 485 486 503
 Van de Sandt H 558 560
 VanDyk A. see Waterman H I 567
 Van Duyn F O 182
 Van Klaveren F W. see György P 183
 Vargha L v 290 297 308
 Vargha L v. see Banga I 104
 Vaubel R. see Stroecker R 318
 Vauthay M 330 334
 Vedder F B. and Rosenberg C 95
 Veen A G van 107
 Veen A G van and Lanzing J C 55
 Vennesland B. see Conant J B 347
 Vennesland B. see Solomon A K 512
 Verbrugge F. see Uber F M 104
 Vermeulen M 566 571
 Verne J. and Mille C 572
 Vertruyen H. see Bezssonoff N 322
 Verzár F 4 8 459
 Verzár F. and Laszt L 186 190
 Verzár F. see Hubner H 179
 Verzár F. see Judowitz M 188
 Verzár F. see Karrer P 477
 Verzár F. see Laszt L 188
 Verzár F. see Pulver R 170
 Vestin R 37
 Vestin R. see Euler H v 195 232 237 580
 Vetter H. see Kuhn R L 10 16 181 221 222
 Vichoever A 455
 Vichoever A. and Cohen I 405 465
 Vigneaud V du Chandler J P Cohn M. and Brown C B 543 544 546
 Vigneaud V du Chandler J P Moyer A W. and Keppel D M 543 546 547
 Vigneaud V du Hofmann K. and Melville D B 470 472
 Vigneaud V du Hofmann K. Melville D B. and György P 471
 Vigneaud V du Hofmann K. Melville D B. and Rachete J R 472
 Vigneaud V du. see Burk D 470
 Vigneaud V du. see György P 470 471 474
 Vigneaud V du. see Hofmann K 472
 Vigneaud V du. see Melville D B 471
 Vilter R W. Schiro H S. and Spies T D 215
 Vilter R W. and Spies T D 239
 Vilter R W. Vilter S P. and Spies T D 192 234 243 244 247
 Vilter R W. see Spies T D 191 192 249 464
 Vilter R W. see Vilter S P 233
 Vilter S P. and Spies T D 212
 Vilter S P. Spies T D. and Mathews A P 241 248
 Vilter S P. Vilter R W. and Spies T D 233
 Vilter S P. see Vilter R W 192 234 243 244 247
 Virgin E. see Euler H v 38
 Virtanen A I. and Laine T 138 263
 Vivanco F 187 188
 Vlodrop C van. see Waterman H I 567
 Voegtlin C. and Chalkley H W 549
 Voegtlin C. see Mayer M E 328
 Vogt E 334
 Vogt Möller P 463
 Vogt Möller F. and Bay F 463
 Vogt R S 570
 Volwiler E H. and Moore M B 591
 Vongersichte E 241
 Vortmann G. see Skraup Z H 224
 Vorwerk J. see Schlubach H 304
 Vrabely V. see Zechmeister L 46
- W
- Wachholder K. and Okrent A 292 319 320
 Wachholder K. and Podesta H H 320
 Wachholder K. see Quensel W 320
 Wackneroder 37
 Waddell J 344 362 594
 Waddell J. and Steenbock H 452
 Waddell J. see Elley H W 599
 Waddell W W. and Guerry D 507
 Wade N J. see Griffith W H 544
 Waerham J 601
 Wagner J R. Axelrod A C. Lipton M A. and Elvehjem C A 183
 Wagner J R. see Kohler G O 627
 Wagner K. H. see Scheunert A 47 43
 Wagner T B 562
 Wagner Jauregg T. and Möller E F 174
 Wagner Jauregg T. and Rauen H 174
 Wagner Jauregg T. see György P 154 155 183 197 574
 Wagner Jauregg T. see Kuhn R 120 154 166 188-190 187 188 192
 Wagtendonk W J van. see Kögl F 475
 Wahl J G. see Day P L 524
 Wahren H 335
 Waissman H A. Mickelsen O. and Elvehjem C A 205
 Waissman H A. Mickelsen O. McAbba J M. and Elvehjem C A 240
 Waissman H A. see Mickelsen O 253 270
 Waissman H A. see Woolley D W 253 255 257 258 260 264 270
 Wäitzmann C 556
 Wald G 90
 Wald G. and Bay H G du 84
 Wald G. and Clark A B 90
 Wald G. and Zussman H 74
 Walden G B 583
 Walker A A. see Spies T D 240 249
 Walker E. see Maguigan W H 365 366
 Walker H A. see Russell W C 85
 Walker O. see Euler H v 53 75
 Walker O. see Karrer P 44 6
 Walker W W. see Dartow E 277 587
 Wallenfels K. see Kuhn R 497 572

- Wallerste n I 573
 Walp L see Hammett F S 549
 Walter E see Re del F 350
 Walters W see S ell A M 507
 Waltz H Gressbach R and Ambros O 594
 Wang Y L and Harr s L J 147
 Wang Y L and Kudkin J 147
 Wanschier O 421
 Warburg E see Elmby A 517
 Warburg O 174 27 58 583
 Warburg O and Christian W 154 172 174 175 179 184 185 20 28 230-233 235 236 586
 Warburg O Christ an W and Grese A 174 179 220 235-238
 Warburg O see Karrer P 238
 Ward G S 553
 Warkany J 418
 Warrat k 591
 Warner E D 506
 Warner E D Brinkhous K M and Smith H P 482 500 507 608
 Warner E D see Brinkhous K M 482 507
 Warner E D see Scanlon G H 506 607
 Warner E D see Smith H P 503
 Warner G C see Went F W 133
 Warner R C see Botsack H 295 298 397
 Waterman H I and Vlodrop C van 567
 Waterman H I Vlodrop C van and V ndyk A 567
 Waterm n R C see Cline J K 105 109
 Waterman R E see Williams R R 101 104 119 126 253 521 575
 Watson E M 463
 Watson E M and T w W P 463
 Watson E M s McArthur C S 456
 Waugh W A Bessey O A and Kug C G 293
 Waugh W A and Kl g C G 293
 Waugh W A see King C G 587
 Wawra C see Lese A J 517
 Wawzonek S l n H M 449
 Webb J L see Leser A J 517
 Webley D M see Quastel J H 142
 Webster A see Fd e C S 99
 Webster C T see H lbrown I M 60-62
 Webster T A and Bourdillon R B 368 370 371
 Webster T A see Angus T C 370
 Webster T A see Ask w F A 369 389
 Webster T A see Bourdillon R B 415
 Webster T A see Ro enhem O 80 343 371
 Webster I M see Smith L I 491
 Wehsl r I S 464
 Weckel K G and Jackson H C 403
 Weekers R see Krause A C 278
 Weermann R A 300
 Wegner M I Booth A N Elvehj m C A and Hart E B 150 194 216 250 271 478
 Wehck H see Karrer P 51
 Weidel H 223-270
 Weidel H and Hazura k 274
 Weidel H and Herz g J 274
 Weidlich G see Windaus A 344 374 387
 Weijlard J 120 580
 Weijlard J and Tauber H 125
 Weijlard J see Eng ls U H 591
 W l Malherbe H 125
 Wehou e S and Kh rasch M S 363
 Weinhouse S see Kharasch M S 595
 Weinmann F 560
 Wentock H H M y E L Arnold A and Price D 265
 Weststock H H Mitchell H K Pratt E P and Williams R J 258 264 267
 Weststock H H s Mitchell H k 256 258 265
 Weststock H H see Williams R J 255 257 260 266
 Weststock M s e He s A P 343 405 418 419
 Wesberg S M and Levin I 18
 Weisrg S M se Myers R P 556
 Weisrger D 334
 Weiss S and Wik us R. W. 140
 Welch A D 540 546 048
 Welch A D and Welch M S 545
 Welch M S see Welch A D 045
 Welker A see Freudenberg A 470
 Wellman J W see Th hler M 169 531
 Wells A W see Taylor H F 564
 Wells P A Lockwood L B Stubbs J J Porg s N and Castrock E A 304
 Wendler N L see Fie er I F 496
 Wendler N I see Th hler M 450 493 436 497 493 500
 Wendt C see Kuhn R 198-203 206 207 209 211 213 214 216 2 584
 Wendt H and Schroeder H 91
 Wenk N see Zeller E A 177
 Wenner W 588
 Went F W Bonner J and Warner G C 133
 Wenz A 119 578
 Werder F v 379 380 407 449 450 606 607 609
 Werder F v and Moll T 451
 Werder F v see Dalmer O 387 605
 Werder F v see Windaus A 350 369 377 39
 Werkman C H see Silverman M 125
 Werkman C H see Wood H G 136 138 139 141 193
 Wertheimer D see Ecker E E 331
 Wertheimer W see Abderhalden E 142
 Wessely L 261
 Wessener J A 554
 Weson D 532
 West P M 134 475
 West P M and Wilson P W 475
 Westenbrink H C k 101 134
 Westenbrink H G K and Goud mit J 128 147
 Westenbrink H G k. Goud smt J and Jansen B C P 147
 Westenbrink H G K and J sen B C P 130
 Westenbrink H G k. see Goud smt J 129 130
 Westphal k 584
 Westphal k. a d Andersag H 577 579 584
 Westphal K see Andersag H 100 108 112 116 118 127 576 577 579

- Westphal K. see Kuhn R 198
202 203 206 207
- Westphal K. see Windaus A
369
- Westphal U 367 413
- Wetter F. see Bock F 346
- Wettstein A. see Karrer P
51
- Weygand F 168 262
- Weygand F. and Burkofer L
172
- Weygand F. and Stocker H
172
- Weygand F. see Kuhn R
158 163 164 166 170 177
407 582
- Wheeler G. A. see Goldberger
J 220 242
- Wheeler G. A. see Sebrell
W H 242
- White E. F. see Beach E. F.
549
- Whittier C. C. 599
- Wiarid P. W. 198
- Wiarid P. W. see Jansen
B C P 102
- Wibaut J. P. see Jansen B. C.
P 102
- Widenbauer F 337
- Widenbauer F. Huhn O. and
Becker G 128
- Widenbauer F. and Koschor
reck K 312
- Wiedling S 284
- Wiegand C. W. see Fieser
L F 497
- Wieland H. and Wishart
R S 277
- Wieland T. see Kuhn R 255
256 260 264-267
- Wieters H. see Dalmer O 587
- Wigelsworth A. E. 556
- Wijk A. van and Reerink E.
H 380
- Wijk A. van see Boer A. G.
344 346 349 359 360 407
595
- Wijk A. van see Reerink E. H.
344 369-371 412 597
- Wild P. see Kipping F. B.
65
- Wildner O. H. M. see Bethke
R. M. 418 419
- Wildner T. S. see Gerstenberger
H. J. 425
- Wildiers E 275 469
- Wiley P. F. see Stiller E. T.
260
- Wilgus H. S. Norris L. C. and
Heuser G. F. 523
- Wilgus H. S. see Norris L. C.
185 191 194
- Wilkie J. B. 81
- Wilkins R. W. see Weiss S.
146
- Wilkinson H. see Aylward
F. X. 7 546
- Wilkinson H. see Beeston A.
W 545
- Wilkinson H. see Gridgeman
N. T. 416
- Wilkinson H. see Pritchard H.
63 72
- Wilkinson J. F. and Ashford
C. A. 334
- Wilkinson J. F. see Portnoy
B. 334 337
- Will G. 92
- Willberg B 318
- Wille E 331 334
- Williams H. H. and Maynard
L. A. 539
- Williams H. H. see Hansen
A. E. 539
- Williams J. K. Lampman
C. E. and Bolin D. W.
96
- Williams R. J. 260
- Williams R. J. Lyman C. M.
Goodyear G. H. Truesdail
T. H. and Holaday D. 253
254 257
- Williams R. J. McAlister
E. D. and Roehm R. R.
266
- Williams R. J. and Major
R. T. 259
- Williams R. J. Mitchell H. K.
Weinstock H. H. and Snell
E. E. 260
- Williams R. J. and Moser R.
255 257
- Williams R. J. Mosher W. A.
and Rohrmann E. 267
- Williams R. J. and Roehm R.
R. 134
- Williams R. J. and Rohrmann
E. 267
- Williams R. J. and Saunders
D. H. 257 266
- Williams R. J. Truesdail
J. H. Weinstock H. H.
Rohrmann E. Lyman C. M.
and McBurney C. H. 255
257 266
- Williams R. J. Weinstock H.
H. Rohrmann E. Truesdail
J. H. Mitchell H. K. and
Meyer C. E. 255 257
- Williams R. J. see Eakin R. E.
212 476
- Williams R. J. see McBurney
C. H. 255 270
- Williams R. J. see Mitchell
H. K. 256 258 263-265
525
- Williams R. J. see Pennington
D. 266 268 270
- Williams R. J. see Snell E. E.
266 268 474 475
- Williams R. J. see Spies T. D.
268
- Williams R. J. see Weinstock
H. H. 268 264 267
- Williams R. R. 33 100 103-
105 110 113 126 577 579
- Williams R. R. Buchman E.
R. and Ruehle E. 105
- Williams R. R. and Chue J.
K. 100 103 116 196
- Williams R. R. and Eddy W.
H. 521
- Williams R. R. and Ruehle
A. E. 113 114
- Williams R. R. Ruehle A. E.
and Finkelstein J. 104
109
- Williams R. R. and Spies T.
D. 99 113
- Williams R. R. and Water-
man R. E. 253 521 575
- Williams R. R. Waterman R.
E. and Keresztesy J. C. 101
119
- Williams R. R. Waterman R.
E. Keresztesy J. C. and
Buchman E. R. 104 126
- Williams R. R. see Buchman
E. R. 111 126
- Williams R. R. see Cline J. K.
103 105 107 109 116
- Williams R. R. see Winter
steiner O. 103
- Williams R. R. see Zama O.
143
- Willmott S. G. and Wokes F.
80
- Willstaedt H. 129
- Willstaedt H. and Jensen H.
B. 72
- Willstaedt K. 56
- Willstätter R. 37 83
- Willstätter R. and Escher H.
H. 39 43
- Willstätter R. and Meig W.
46 50
- Willstätter R. and Sobotka
H. 554
- Willstätter R. and Stoll A. 44
51 52
- Wilson C. W. 516
- Wilson H. E. C. and Roy G.
K. 211 214
- Wilson L. T. see Light R. F.
419
- Wilson P. W. see West P. M.
475
- Wilson W. R. see Hansen A.
E. 539
- Winckel M. 554
- Winckelmann H. 324
- Windaus A. 343 346 374 595
597 599 604
- Windaus A. and Auhagen E.
377
- Windaus A. and Bock F. 344
405
- Windaus A. and Borgeaud P.
357
- Windaus A. and Brunken J.
356

- Windaus A and Buchholz K 363
 Windaus A Deppe M and Roosen Runge C 400
 Windaus A Deppe M and Wunderlich W 379 374 376 379 383 400
 Windaus A and Dmroth K 399
 Windaus A Dithmar K and Fernholz E 375 391
 Windaus A Gaede J Köser J and Stein G 381
 Windaus A and Grundmann W 396
 Windaus A and Güntzel B 372 376 379 383
 Windaus A and Hess A 343
 Windaus A Inhoffen H H and Reichel S v 354
 Windaus A and Kaufmann O 363
 Windaus A and Kuhr E 409
 Windaus A and Langer R 344 349 363
 Windaus A Lettré H and Schenck F 344 360
 Windaus A Linsal and Buchholz K 349 364 407
 Windaus A and Linsert O 350
 Windaus A Linsert O and Eckhardt H J 361 362
 Windaus A Linsert O Lüttringhaus A and Weidlich G 344 374 387
 Windaus A and Lüttringhaus A 350 354 356 374
 Windaus A Lüttringhaus A and Busse P 380
 Windaus A Lüttringhaus A and Deppe M 374
 Windaus A and Nagatz J 349 363
 Windaus A and Roosen Runge C 393
 Windaus A and Rygh O 408 418
 Windaus A and Schenck F 395 604
 Windaus A and Thiele W 393
 Windaus A and Trautmann G 390 400
 Windaus A Tschsche R and Grewe R 105 109
 Windaus A Tschesche R Rubkopf H Laquer F and Schultz P 100 102
 Windaus A Werder F v and Gachard B 350 392
 Windaus A Werder F v and Lüttringhaus A 377
 Windaus A Westphal K, Werder F v and Rygh O 369
 Winegar A H Parson P B and Schmidt H 250
 Winkel M 559
 Winter L B 276
 Winterstein A 41 45
 Winterstein A see Kuhn R 46 50
 Wintetser O and Bergström S 360
 Wintersteiner O and Ritzmann J R 360
 Wintersteiner O Williams R R and Ruehle A E 103
 Wintrobe M M 214
 Winzler R J see Burk D 475
 Witek A M see Bills C E 419
 Wirth T see Elmby A 335
 Wisansky W A and Ansbacher S 165
 Wianky W A Martin G J and Ansbacher S 285
 Wisansky W A Martin G J Tchiowski C T and Ansbacher S 285 286
 Wisansky W A see Martin G J 85
 Wischnegradski A 224
 Wishart R S see Wieland H 477
 Wisnicky W see Coombes A I 55
 Withrow R B see Spert G 598
 Woelfel E A see Holmes A D 326
 Wörner A and Kuelwein F 597
 Woessner W W Elvehjem C A and Schuette H A 317 318
 Wohlbach S B 330
 Wohlbach S B and Howe P R 330
 Wohlfart O see Ekblad M 462
 Wohmann M 110
 Woker G and Bernhard P 536
 Wokes F see Willmott S G 80
 Wolbach S B 27
 Wolbach S B and Beey O A 94
 Wolbach S B and Howe P R 92 93
 Wolbach S B see Bessey O A 83 189 191
 Wolbach S B see Blackfan K D 95
 Wolbach S B see Sohl A T 414 421
 Wolf E J 571
 Wolf J 559 566
 Wolff 83
 Wolff K and Havemann R 603
 Wolff L K 413
 van 79
 Wolff L F see Bruns H R 6
 Wolff K L see Eckelen M
 Wolfenstein R 565
 Wolter E see Auwers F v 356
 Wood E L and Hues H M 462
 Wood H G Anderson A A and Werkman C H 193
 Wood H G and Werkman C H 136 138 139
 Wood H G Werkman C H Hemingway A and Nier A O 139 141
 Woods A W see Spas T D 240 249
 Woods D D 263 285
 Woods D D and Fides P 283 285
 Woods E see Owens H S 269 54
 Wooley J G 11
 Wooley J G see Sebrell W H 193
 Woolley D W 259 264 275 276 278 280
 Woolley D W and McCarter J R 504 509
 Woolley D W Strong F M Madden R J and Elvehjem C A 239
 Woolley D W Waisman H A and Elvehjem C A 255 258 260
 Woolley D W Waisman H A Mickelsen O and Elvehjem C A 253 257 264 270
 Woolley D W see Elvehjem C A 220 242 250
 Woolley D W see Hutchings B L 212
 Worden A N see Chek H 198 199 214
 Work C E see Flisadr M M 183 197 264
 Work L T see Boober L E 580
 Work T S see Bergel F 441 443 445
 Work T S see Todd A R 437 438 443
 Worm H A 71
 Wortis H see Bueding E 148
 Wright I S 335
 Wright I S and Lienfeld A 336
 Wright I S Lienfeld A and MacLennan E 325
 Wright I S see Lienfeld A 320

Wright L D see Snell F F
24 260 474
Wright M D and Drummond
J C 449 457
Wright M D see Baker A Z
416
Wright M D see Drummond
J C 460
Wulff H J see Negelein E
299
Wunderlich W 303
Wunderlich W see Windaus
A 372 374 376 377 383
400
Wurmser R 200
Wyatt W R 291 290
Wyeno J see Landy M 283
Wyss O 983

Y

Yakuzi I see Okunuki K
174
Yamamoto K see Kobayashi
L 80
Yamamoto R and Muraoka
T 44
Yamamoto R and Tin S
41
Yap K S see Karrer P
400
Yoder L 409 605
Yoder L Thomas B H and
Lyons M 409
Yoder L see Eck J C 409
Yokata K see Katmatsu S
225
Yoshida R K 279

Yousmans J B and Corlette
N B 92
Young F C see Smith E L
39 60 71 81
Young F H and Robinson
H D 563
Young J 403
Young L 278 273
Youngberg C E 321
Yudkin J see Wang Y L
147

Z

Zach C E 515
Zbinden C see Bakke A 253
Zecher G 601
Zechmeister L 50
Zechmeister I and Chohnokv
J v 41 51
Zechmeister I Chohnoky L
v and Vrabely V 46
Zehmetter L Karrer P and
Kuhn R 37
Zechmeister L and Tuzson P
38
Zehnder F see Fuler H v
331
Zeller F A Stern R and
Wenk N 177
Zentmire J see Jeans I C
82 90
Zerfas L G see Dixon M
297
Ziffern S E Owen C A
Hoffman G R and Smith
H P 508
Ziffern S E see Smith H P
507 508
Zilva S S 290 291 293 16
Zilva S S see Arcus C L
290 291 298
Zilva S S see Connell J B
290
Zilva S S see Harden A
290
Zilva S S see Snow G A
392
Zima O 116 576 577 389
Zima O Ritsert K and Moll
T 143
Zima O and Williams R R
143
Zimmerli A 594
Zimmerli A see Nield C H
413
Zimmermann W and Franken
burger W 597
Zmachinsky A see Ellis I N
190
Zöller F see Hentschel H
496
Zscheile I P see Hoagness
T R 30
Zubrys A see Fuler H v 34
72 73
Zucker T F 387 401 56 601
Zucker T F Pappenheimer
A M and Barnett M 743
Zucker T F see Simons E J
H 344 387 383
Zuckerman I C Kogut B
Jacobi M and Cohen J Y
507
Zusman H see Wald G 74
Zwemer K L see Jaegerblut
C W 331

SUBJECT INDEX

[**ABBREVIATIONS** acty (activity) defn (definition) defcy (deficiency) dtn (determination) dehyd (dehydrogenation) hyd (hydrogenation) vdn (validation) provit (provitamin) vit (vitamin)]

A

- Abortion v t F ther py 463
- Absorption constant lefn °2
- Absorption spectrum detn 21 unts °1-°2
- Accessory factors 12
- Acetaldehyde enzymat c hyd (n) 227 29
- Acetamide ethyl as solvent for v t D 403
- Acetaminine in vit B₁ synthesis 106 107 108 122
- Acet c ac d synthesis from pyruvic acid 139 140
- Acetamido-ethyl ether in vit B₁ synthesis 106
- Acetoacetic ac d enzymat c hyd (n) 227 synthesis in organism 141
- α Aceto butyro lactone synthesis 111
- α Aceto α chloro butyro lactone synthesis 111 11
- γ Aceto-γ chloro propyl acetate in vit B₁ synthesis 112
- γ Aceto-propyl acetate in vit B₁ synthesis 112
- Acetylcholine 546 in nerve excitement 14°
- Acetylcholine estera c 14°
- *Acetyl thiam n nerve excitement from 142
- Achromotrichia n β amino-benzoic acid defcy 286 in pantothenic acid defcy 269
- Acid ba c propert of d et in rickets 409 425
- Acidos s in vit D defcy 425
- Acne vulgar s biotin therapy 477
- Acrodyn a in vit B₁ defcy 198 213
- Acrodynia ratte t for v t B 211
- Activation of provits D to vits D 368
- Adaptometer 93
- Addison s dise se vit C therapy 334
- Adenine vit B acty 5 2
- Adenine triphosphate 125
- Adenosine 5 monophosphoric cid 179
- Adenos n triphosphoric acid 173
- Adenylic acid 125 179 (vit B) 5 2
- Ad nyl pyrophosphate 125
- Aderna 197
- Adpic acid from biotin 47°
- ADMA Un t of vit D 417
- Adrenal cortex xamination for v t C d fcy 337
- Adrenal cortex hormones relation to vit B 145 relation to vit C 332
- Adrenal hemorrh ge in pantothenic acid defcy °63
- Adrenal ne β amino benzoic acid effect 28, 286
- Advitant 13
- Agng premature in pantothenic acid defcy 2, 10 n riboflav n d fcy 131
- β Alanine biogenesis 63 n p n othenic acid °8
- pant thenic a d acty 264 syntle 6°
- Alcohol enzymatic dehyd (n) 174 ° 7
- Aldehydes enzymat ox d n 176
- Aldehyde mutation nicot nam de enzymes relat on 2 7
- Aldehyde oxid c 171
- Algin c ac d growth factor 54°
- Allergy vit C therapy 334
- Alloxan n riboflav n syntle s 163
- Alloxan ne 160 synthesis 173
- 2 Allyl 1 4 naphthoquinone vit K acty 496
- o Allyl phenol vit E acty 451
- Almqvist Reference Stand rd of vit K 504
- Alopecia in noitol defcy 280 in riboflav n defcy 170
- Alum hum chlor de test for vit A 80 for vit D 412
- Amadori rearrangement 179 °
- β Amino acet nlide in te t for vit B₁ 129
- β Amino acetophenone in te t for vit B₁ 19
- Amino acid s essential 540
- Amno ac d metabolism n n cotin c acid biosen sis 2 6
- nicotinic acid r lat on 244 245
- vit B₁ relat on 188
- v t B relation 213
- vit C relat on 331
- vit F relation 4 7
- d Amino c d oxida c 171
- d Amno acid o dase t st for riboflav n adenine-d nucl otid 184
- d Amino-ac d ox d tion 175
- β Am no-o allyl phenol v t E acty 451
- β Amino-benzo c acid 283
- β Am no benzoic acid acetate 28
- α Am no β hyd oxy butyr c a d (threonine) 540
- α Amino iso-c proic acid (leucine) 540
- α Am no isovaleric acid (valine) 541
- Am no methylene m lonic n trile 106

- 2 Amino 4 methyl 5 β hydroxy ethyl thiazole synthesis 122
- 4 Amino 2 methyl 1 naphthol hydrochloride 494
vit K acty 499
- 4 Amino-3 methyl 1 naphthol hydrochloride vit K acty 499
- α Amino β (4 methyl thiazole 5) propionic acid 119
- α Amino β methyl valeric acid (isoleucine) 540
- 4 Amino pyrimidine 10a
- δ Amino valeric acid in nicotinic acid biogenesis 226
- Amylase activation by vit C 328
- Anaphylaxis vit C therapy 333
- Anderson Nightingale dilution test 78
- Anemia in extrinsic factor defcy 527
in lysine defcy 541
in nicotinic acid defcy 246
in vit B₁ defcy 214 21a
in vit B₂ defcy 523
in vit C defcy 333
in vit M defcy 595
- Aneurin 99
- Aniline test for vit D 413
- Anorexia biotin therapy 477
vit B₁ therapy 146
vit F therapy 464
- Ansbacher Unit of vit K 504
- Antagonism of vitamins 29
- Anti acrodynia rat factor (vit B₆) 197
- Antialopeia factor for mice (inositol) 275
- Anti beriberi vit (vit B₁) 99
- Anticanceric factor 283
- Antidermatosis vit (pantothenic acid) 253
- Anti egg white injury factor 469
- Anti encephalomalacia vit 435
- Anti gray hair factor 283
- Antihemorrhagic vit 481
- Antimony trichloride test for provit D 368
for vit A 78
for vit D 413
- Antineuritic vit 99
- Antioxidant for vit A 68
- Antioxidative properties of vit E 440
- Anti pernicious anemia factor 527
- Anti perosis vit (vit B₂) 593
- Anti pneumonia factor 523
- Antirachitic vit 341
- Antiscorbutic vit 289
- Antiskorbutin 289
- Anti spectacled eye factor for rats 269 280 477
- Anti sterility factor 435
- Anti tetany compound No 10 379
- Antixerophthalmic vit 57
- A.O.A.C Unit of vit D 417
- Aphanicin constitution 42 49
isolation 45
properties 42
vit A acty 74
- Aphanin constitution 42 49
isolation 45
occurrence 39
properties 42
- Apoccarboxylase 123 125 126
- β Apo 2 carotinal preparation 76
provit A action 76
vit A acty 76
- β Apo-2 carotinal absorption spectrum 69
preparation 76
provit A action 76
- β Apo 3 carotinal 69
- β Apo 4 carotinal 69
- β Apo 5 carotinal 69
- β Apo 6 carotinal 69
- Apoenzyme 171 227
- Appetite in vit B₁ deficiency 146
- Aquoflavin 187
- Arabinose growth factor 549
- d Arabo ascorbic acid vit C acty 314
- Arachidonic acid 531
biogenesis 535
constitution 535
essential fatty acid acty 536
isolation 533
occurrence 539
- Arginase activation by vit C 328
- Arginine essential amino acid 540
vit B₁ action 52
- Ariboflavinosis 191
- Arsenic chloride color reaction for vit A 80
- Arsenic poisoning vit C therapy 330 334
- Arsenocholine as essential methyl group sup plier 546
- Arthritis vit C therapy 334
- Ascorbic acid (l ascorbic acid) 289
cellular oxidn 327
classification 290
combined form 292
nomenclature of derivatives 289
determination in presence of dehydro ascorbic acid 322
homologs vit C acty 314
separation from dehydro ascorbic acid 392
stereoisomers vit C acty 314
- Ascorbic acid oxidase 327
- Ascorbic acid oxidase test for vit C 322
- Ascorbigen constitution 301
isolation 293
occurrence 292
- Aspartic acid decarboxylation 463
- Astaxanthin 74
- Asthma p amino-benzoic acid effect 286
- Ataxia in vit B₁ defcy 146
- A T 10 379
- Avidin 476
- Avitaminosis defn 31
- Axerophthol 57
- Azelais aldehyde carboxylic acid from linolenic acid 534
- Azelais acid from linoleic acid 534
- Azo test for vit C 321

B

- Bachstez Cavallini test for vit C 320
- Bacteria test for p amino benzoic acid 284
for biotin 474
for nicotinic acid 242
for pantothenic acid 266
for riboflavin 184
for vit B₁ 211
- Baldness biotin therapy 477
riboflavin therapy 190
- Barac's test for vit C 321
- Barbituric acid in riboflavin synthesis 170
- Bearer protein 171 227

- Beer's law 21
Benzene 1 2 3 4 tetracarboxylic acid 357
Benzimidazole compounds synthesis 167
Berberin in pantothenic acid deficiency 268
 in vit. B₁ deficiency 146
 in vit. B deficiency 192
Betaine 543
 properties 544
Bile ducts vit. A deficiency due to obliteration of 93
Biliary fistula vit. K therapy 507
Biocatalysts vitamins as 14
Biochemical methods for the determination of vitamins 22
Biological methods for the determination of vitamins 22 23
Bios 11 275
Bios 1 270
Bios 11 75 469
Bios 11 b (biotin) 469
Biosterol 57
Biotin 469
 optical activity 471
 protein complex 476
 solubility 471
4,5-Bis-desoxy vit. B₆ vit. B activity 409
Blacktongue in nicotinic acid deficiency 247
 in riboflavin deficiency 19
Bladder stones in vit. A deficiency 92
Bleeding in vit. K deficiency 507
 vit. C therapy 334
Bleeding time determination 507
Blindness in vit. A deficiency 9
Blood coagulation theory of process 505
Blood clotting time determination 50 508
Blood level of calcium in vit. D deficiency 4.0 4.3
 4.4 4.5 428
 of calcitonin 85 93
 of carboxylase 149
 of coenzyme A 244 249
 of nicotinic acid 44 246 249
 of pantothenic acid 268 270
 of phosphatase in vit. D deficiency 423
 of phosphorus in vit. D deficiency 4.0 4.23 4.24
 4.25 4.28
 of provitamin A 85 93
 of riboflavin 187
 of riboflavin-dinucleotide 187
 of vit. A 85 93
 of vit. B₁ 133 149
 of vit. B 112
 of vit. C 3 5 326 333-335
 of vit. D 418
 of vit. E 456 464
Blood phosphatase test for vit. D deficiency 429
Blood pressure vit. B₁ action 146
 vit. P action 517
Blue unit (Moores) of vit. A 83
Bomskov Unit of vit. E 453
Bone decalcification in D hypervitaminosis 429
 elasticity in vit. D deficiency 428
 fracture in vit. C deficiency 333
 fragility in A hypervitaminosis 95
 growth in vit. D deficiency 420 421
 pain in vit. C deficiency 333
Bourquin Sherman Unit of riboflavin 184
Bradford test for vit. B₁ 131 147
 α -Bromo-aceto-acetic ester in vit. B₁ synthesis 111
Bromoethyl acetate in vit. A synthesis 63 64
 α -Bromopropionic acid ester in vit. B synthesis 207
B S Unit for riboflavin 183
- ### C
- Caecothel test for vit. C 3 2
Cadaverin enzymatic oxidation 177
Calciferol 341
Calcification in vit. D deficiency 427
Calcium absorption in vit. D deficiency 4
Calcium metabolism parathyroid action 426
 vit. C action 330
 vit. D action 4.0 4.2 4.24 4.25 4.29
 vit. P action 517
Calculi urinary in vit. A deficiency 9
Canal rays for activation of provitamin D 371
Capillary fragility in vit. P deficiency 517 518
Capillary fragility test 336
Capillary permeability in vit. P deficiency 517 518
Capillary resistance in vit. C deficiency 336
 in vit. P deficiency 336 17
n-Capric acid from linolenic acid 534
Carbohydrates 54
Carbohydrate metabolism in nicotinic acid relation 7 45
 vit. A relation 88
 vit. B relation 135
 vit. B relation 171 188
 vit. C relation 331
 vit. D relation 4 6
Carboxylase constitution 123
 isolation 123
 system 173
Carboxylation of pyruvic acid 137
Cardiovascular dysfunction in vit. B deficiency 146
Caries dental in vit. D deficiency 4.23
Carnosine hydrolysis to β -alanine 763
Carotenes 54
 α Carotene absorption spectrum 40
 constitution 41 46
 isolation 44
 occurrence 38
 optical activity 41 43
 properties 41
 vit. A activity 74
 α β Carotene 41 46
 β Carotene absorption spectrum 40 43
 constitution 41 46
 determination 53
 isolation 44
 occurrence 38
 properties 41
 vit. A activity 74
 β β Carotene 41 46
 γ Carotene absorption spectrum 40
 constitution 41 46
 isolation 44
 properties 41
Carotene oxide vit. A activity 77
Carotene level of blood 33
Carotenoids 46
 separation 57
 β Carotene-oxide preparation 70
 vit. A activity 75
Carr Price method for determination of vit. A 78
Carr Price value of vit. A 78 83

- Cartilage growth factor 542
 Catalase activation by vit C 328
 Cataract in riboflavin deficiency 190
 in tryptophan deficiency 541
 in vit C deficiency 333 334
 Cataractulin test for vit B₁ 131
 Cathepsin activation by vit C 328
 Cathode rays for activation of provit D 371
 Celiac disease vit H deficiency due to 93
 Cevitamic acid 289
 Chase Sherman Unit of vit B₁ 132
 Chaulmoogric acid essential fatty acid acty 536
 Cheilosis in riboflavin deficiency 191
 Chemical methods for the detn of vitamins 22
 Chick antidermatitis factor 253
 Chick antipellagra factor 253
 Chick test for *p* amino benzoic acid 284
 for biotin 474
 for pantothenic acid 266
 for riboflavin 184
 for vit D 415
 for vit K 302
 Chick unit of vit D defn 384 407 410
 Chilblains vit B₁ deficiency 215
 Chloralhydrate test for provit D 367
 Chloric acid color reaction for vit A 80
 γ Chloro- γ aceto propyl alcohol 112
 γ Chloro γ aceto-propyl alcohol and derivatives 116
 2 Chloro-4 methyl 5 acetoxy-ethylthiazole syn thesis 113
 Chloroflavin 161
 Cholestane 331
 Cholesterlene sulfonic acid vit D acty 409
 Cholesterol 350 351
 biogenesis 366
 dehydn to 7 dehydro cholesterol 362
 in 7 dehydro cholesterol synthesis 360 361
 Cholesterol metabolism vit A relation 88
 Choline 543
 biogenesis 546
 properties 544
 synthesis by transmethylation 547
 Chondroitin growth factor 542
 Chondroitin sulfuric acid pantothenic acid acty 264
 Chroman vit F acty 451
 Chroman ring system in vit E 442
 Chromatolysis in vit E deficiency 459
 Chromotrichia factor 283
 Chromous chloride method 65
 Cirrhosis of the liver in essential methyl group deficiency 544
cis Aconic acid in citric acid cycle 137
cis trans Isomerism of vit A 65 71
 Citric acid enzymatic dehydri 174 227
 synthesis from oxaloacetic acid 137
 synthesis from pyruvic acid 137 138
 vit D acty 409 425
 Citric acid cycle 137
 Citrin (vit P) 513
 C L O Unit of vit A 78
 Clinical Unit of vit D 417
 Coagulation vitamin 481
 Cocarboxylase dephosphorylation 124
 detn 131
 hydrolysis 124
 identification 123
 in blood cells 133
 occurrence 101
 protein complex 135
 synthesis 124 125
 vit B₁ acty 126
 Cod Liver Oil Unit of vit A 78
 Codehydrogenases oxidn of the dihydro-com pounds 172 174
 Codehydrogenases I and II requirements 200
 Codehydrogenase I 229
 absorption spectrum 230 231 233
 constitution 232
 detn 233 234
 isolation 230
 occurrence 229
 optical acty 231
 properties 230
 separation from codehydrogenase II 233
 separation from riboflavin adenine-dinucleo tide 230
 standard 234
 synonyms 229
 synthesis 233
 Codehydrogenase II 234
 absorption spectrum 231 233 233
 constitution 236
 detn 237
 isolation 235
 occurrence 234
 optical acty 236
 properties 235
 separation from codehydrogenase I 233
 separation from riboflavin adenine dinu cleotide 230
 synonyms 234
 synthesis 237
 Coenzyme 171 227
 Coenzyme R (biotin) 469
 Coenzyme R test for biotin 474
 Coenzyme I 229
 Coenzyme factor 173
 Coferment I 229
 Coferment of fermentation 229
 Colitis vit K therapy 407
 vit M therapy 525
 Colostrinum bulgicum test for biotin 474
 Colpokeratosis in vit A deficiency 92
 Complement vit C relation 331
 Complementary factor 197
 Compound specificity defn 19 406
 of vit D 408
 Coniferyl alcohol in biogenesis of flavanones 515
 Constant yield oil 61
 Convulsions in vit B₁ deficiency 146 147
 in vit B₁ deficiency 214
 in vit E deficiency 469
 Coreductase 229
 Cornea vascularization in riboflavin deficiency 189
 131
 Cornell Unit of riboflavin 185
 Corpuscular rays for activation of provit D 371
 Cqumaran ring system and vit E structure 442
 Coward Unit of vit D 417
 Cowgill Unit of vit B₁ 133
 Cozymase 229
 Creatine essential methyl group supplier 543
 metabolism in vit E deficiency 467 464
 synthesis by transmethylation 547

- Cretinism *n vit E* defcy 400
 Crotyl alcohol enzymatic reduction 177
 Cryptoxanthene constitut on 41 48
 isolation 45
 occurrence 39
 prop rties 41
 ψ Cumoquinol from β tocopherol 443
 from γ tocopherol 444
 Cumo tocopherol 436
 Cyanin dy test for *vit B* 210
 Cyano- cetamide *n vit B₆* synthes s 404
 3 Cyano 4 ethoxy methyl 6 methyl 2
 pyridone 204
 3 Cyano 4 ethoxy methyl 5 nit o 6
 m thyl pyridone 204
 Cyanic ethyl acet te t for *vit B* 501
 Cyanogen bromide test for a cotin c acid 40
 in urine 248
 in blood 49
 Cyano-succinate in *vit B₁* synthe s 108
 1 (β Cyclo-geranyl) geramol *vit A* acty 75
 Cyclohe anebexol 270
 Cytein enzymatic o dn to cystine 228
 v tagen nature 549
 Cytochromes a b and c enzym tic reduction of
 o dized 177
 Cytochrome a 174
 Cytochrome b 174
 Cytochrome c 173
 in *vit C* oxid 327
 - Cytochrome c oxidase 173
 activat on by *vit C* 329
 in *vit C* oxid 377
 Cytochrome c r ductase 171
 Cytoflav 154 177
 Cytopenia in *vit M* defcy 525
- D
- Dam Unit of *vit B* 504
 Dann Evelyn L 620 $m\mu$ value 78
 Dann Unit of *vit K* 504
 Daphn a test for *vit E* 450
 Dark adaptation in *vit A* d fcy 89 9 93
 in *vit B₁* defcy 197
 test 93
 Deafn e ghth nerve (high ton) nicot nic acid
 tr alment 247
 Decalcification in *D* hypervit mino s 4 9
 Decarboxyl tion of α keto butyric ac d 136
 of α keto- ϵ rboxylic ac d 136
 of α k to-glutaric ac d 136
 of α keto valeric acid 136
 of pyruvic ac d 136
 D hydro-ascorb c acid cellular reduct on 327
 detn 316
 detn in pr en e of ascorb c ac d 322
 occurrence 291
 preparation 98
 separation from ascorbic acid 372
 vit C acty 314
 Dehydro-cholestenone 363
 7 Dehydro-chole t rol activated 341
 activat on 363
 b og n sis 366
 const tution 359
 occurrence 345
 propert es 349
 synthes s 360
 D hydro-ergo terol from ergosterol 399
 from π opyro calc ferol 399
 Dehydrog nases 227
 Dehydro lumisterol from lumisterol 399
 from pyro calc ferol 399
 Dehydro neoergosterol 307 358
 β Dehydro semicarotenone prepar t on 75
 vit A acty 75
 7 Dehydro tosterol activat d 341
 propert s 349
 synthesis 360
 7 Dehydro tigmasterol propert es 349
 synthesis 360
 dl 3 4 D hydro α tocopherol *vit E* acty 450
 Demethyl dihydro-calcif rol 383
 Dentine apposition in *vit A* d fcy 93
 Dephosphorylat on en ymat c l 4
 of cocarboxyla e 130
 Depigment t on in β am no-benzoic ac d defcy
 786
 in β n tothenic ac d defcy 279
 Dermatitis in b otin d fcy 476 477
 in ribofl v n d fcy 191
 Dermato s in essential fatty acid defcy 539
 in *vit A* defcy 97
 6 Desoxy l asco bic ac d *vit C* acty 314
 6 Desoxy tocol *vit E* acty 449
 4 Desoxy *vit B* *vit B₆* acty 209
 Detoxific tions by *vit B₁* 146
 by *vit C* 330
 by *vit P* 517
 Deutero leuco-ribofl n 1 8
 D xtrose n it C b ogenes s 312
 γ γ Diac to- γ halog no-propyl alcohol 117
 γ γ Diaceto γ merc pto propyl alcohol 117
 Diacetyl from α tocopherol 441
 2 3 Disallyl 1 4 naphthoquinone *vit K* acty 497
 Diam n s en ymat c oxidn 176
 Diam ne ox dase 171
 α D amino caproic ac d (lysin) 540
 Di phora 173
 D phora 1 171
 D pho a e II 171
 Diapho ases classification 174
 dehydn of the r duc d 174
 hyd n 174
 Diaphragm metabolism in riboflav n defcy 190
 Diarrhea *n vit M* defcy 5 5
 D o zo benzene sulfu ic acid n test for *vit B*
 1 8
 2 2 Di n butyl-chr man *vit F* a ty 451
 5 5 D chlo o-barb turic ac d in r boflavin syn
 thesis 169
 4 D chloro benzene d zon um chlor de in test
 for *vit B₁* 129
 2 6 Dichloro-ph nol ndophenol te t for *vit C*
 316
 2 6 D chlo oquinone chlorim de test for *vit B₆*
 210
 2 9 Diethyl chroman *vit E* acty 451
 Diethyl m thyl tocol *vit E* acty 449
 5 7 D ethyl tocol *vit I* acty 449
 D lton n pr p tation of t rals 3 2
 D o h enyl ph nol *vit E* acty 451
 Dihydro arach donic ac d 538
 α D hydro-caroten *vit A* acty 70
 β D hydro carotene *vit A* acty 75

- Dihydro-cocarboxylase vit B₁ acty 126
 Dihydro-codehydrogenases dehyd_n 229
 Dihydro-codehydrogenase I absorption spectrum 230 231 233
 fluorescence 231 233
 occurrence 230
 Dihydro codehydrogenase II absorption spec-
 trum 231 233 235
 fluorescence 235
 α Dihydro-ergosterol occurrence 365
 2^o Dihydro-ergosterol activated 341
 constitution 359
 properties 349
 synthesis 363
 Dihydro nicotinamide compounds 238
 2 (β γ Dihydro-phytyl) 14 naphthoquinone vit
 K acty 496
 Dihydro riboflavin 160 180
 Dihydro-tachysterol 379
 Dihydro-vit B₁ vit B₁ acty 126
 Dihydro vit D₁ from tachysterol 379
 from vit D₂ 379 392 393
 58 Dihydro-vit K₁ vit K acty 499
 α β Dihydroxy γ butyrolactone pantothenic acid
 acty of condensation product with β alanine
 265
 33 Dihydroxy α carotene 44
 α γ Dihydroxy β β dimethyl butyric acid 259
 α γ Dihydroxy β β dimethyl butyryl β
 alanide 254
 Dihydroxy maleic acid 297
 67 Dimethyl alloxazine 160
 12 Dimethyl 4 amino 5 methyl amino-
 benzene 163
 45 Dimethyl 2 amino phenyl ribamine 164
 25 Dimethyl-4 amino pyrimidine synthesis 109
 67 Dimethyl 9 (δ 1 arabityl) isalloxazine 181
 67 Dimethyl 9 (I 1 arabityl) isalloxazine 181
 25 Dimethyl benzoquinone vit K acty 498
 11 Dimethyl 3 *trans* butyl 14 dihydro anthraqui-
 none vit K acty 497
 25-Dimethyl-4 chloro-pyrimidine synthesis 109
 57 Dimethyl 8 ethyl tocol vit E acty 449
 α α Dimethyl glutaric acid from β carotene 48
 24 Dimethyl 3 hydroxy 5 hydroxy
 methyl pyridine 209
 α α Dimethyl β hydroxy propionic acid
 in pantothenic acid 259
 25 Dimethyl 4 hydroxy pyrimidine synthesis
 109
 Dimethyl maleic anhydride from α tocopherol
 441
 Dimethyl malonic acid from β carotene 48
 16 Dimethyl naphthalene from vit A 6^o
 23 Dimethyl 14 naphthohydroquinone sodium
 disulfate vit K acty 498
 23 Dimethyl 14 naphthoquinone vit K acty
 496
 25 Dimethyl 14 naphthoquinone vit K acty
 497
 26 Dimethyl 14 naphthoquinone vit K
 acty 497
 27 Dimethyl 14 naphthoquinone vit K acty
 497
 28 Dimethyl 14 naphthoquinone vit K acty
 497
 67 Dimethyl 14 naphthoquinone vit K acty
 497
 23 Dimethyl 14 naphthoquinone oxide vit K
 acty 498
 27 Dimethyl 14 naphthoquinone oxide vit K
 acty 498
 26 Dimethyl-3 phytol 14 naphthoquinone vit
 K acty 497
 67 Dimethyl 9 (δ 1 ribityl) iso-alloxazine 153
 α Dimethyl succinic acid from β carotene 48
 23 Dimethyl 5 6 7 8 tetrahydro 14 naphtho-
 quinone vit K acty 451
 58 Dimethyl tocol 436
 78 Dimethyl tocol 436
 24 Dinitro-chlorobenzene test for nicotinic acid
 241
 in urine 248
 Dioxido stearic acid essential fatty acid acty
 536
 Dioxo acetone phosphate enzymatic dehyd_n
 174
 Diphosphoglycerate enzymatic hyd_n 227
 Diphosphopyridine nucleotide 229
 Diphosphothiamin 123
 Diphosphothiamin magnesium protein 123
 m Dipyrityl in nicotinic acid synthesis 224
 Distillation short path high vacuum 61
 of vit A 61 67
 of vit A₂ 68 70
 of vit D 383
 of vit E 438 448
 Dithioformate 118
 Docosa hexa-enoic acid essential fatty acid acty
 536
 Docosa penta enoic acid 538
 2 Dodecyl 2 5 7 8 tetramethyl 6-oxychroman vit
 E acty 450
 Drug hypersensitivity vit C therapy 334
 Duro hydroquinone vit E acty 450
 Duroquinone from α tocopherol 441
 vit K acty 498
 Dysentery in vit M defcy 525

E

- Echinenone constitution 42 48
 isolation 45
 occurrence 39
 properties 42
 Eczema essential fatty acid therapy 539
 Edema in vit B₁ defcy 142 146
 Egg hatchability in pantothenic acid defcy 489
 in riboflavin defcy 191
 in vit D defcy 420
 in vit E defcy 463
 Fgg production in choline defcy 545
 in riboflavin defcy 191
 Egg white toxicity 476
 α Eicosamic acid from arachidonic acid 535
 Electrons for activation of provit D 371
 Elimination curve 61
 Eluate factor (vit B₁) 197
 Emaciation in biotin defcy 476
 Enamel organ in vit A defcy 93
 Encephalomalacia in vit E defcy 463
 Endurance lowered in vit B₁ defcy 146
 Enriched flour vit content 34
 Enzymes β amino benzoic acid effect 285
 ascorbic acid effect 328
 Epi-cholesterol 363

- Epi 7 dehydro-cholesterol constitution 359
 properties 349
 synthesis 363
 Epi- α gosterol constitution 359
 properties 348
 synthesis 363
 Epilepsy idopathic in vit B₁ defcy 214 215
 Epithelium atrophy in vit A d fcy 91
 Epithelium protecting vit 57
 Ergas 14
 Ergons 15
 Ergostadi acetol 354 355
 Ergostadiolone 36 354 355
 Ergostane 350 351
 Ergostane triol-3 56 354 355
 Ergostanol 350 351
 Ergostanyl chloride 350 351
 Ergostene-dione 354 355
 Ergosterol absorption spectrum 350
 activated 341
 activation 368
 biogenesis 365
 constitution 350
 maleic anhydride addition product 356 357
 occurrence 345
 optical acty 348
 propertie 348
 Ergosterone 363
 Eriodictin 513-518
 Eriodictyol 513-518
 Essential amino acids 540
 Essential carbohydrates 542
 Essential fatty acids 531
 Essential methyl group 543
 Essential sulphydryl group 549
 Essential sulfur compounds 549
 Esterase in vit C defcy 329
 Ethoxy acetyl acetone in vit B₁ synthesis 204
 Ethoxy ethyl propionate in vit B synthesis 107
 Ethoxy methylene malonic nitrile in vit B₁ synthesis 106
 Ethyl format in vit B₁ synthesis 107
 6 Ethyl 7 methyl 9 (d 1 rib ty) isomalloxane 181
 2 Ethyl 14 naphthoquinone vit K acty 496
 2 Ethyl 3 phytol 14 naphthoquinone vit K acty 497
 3 Ethyl pyridine in nicotinic acid synthesis 274
 7 Ethyl 9 (d 1 rib ty) isomalloxane 181
 Excretion of fat in nicotinic acid deficiency 248
 Exogenous hormones 13
 Extracellular factor in pernicious anemia 57
 Eye lesions in tryptophane deficiency 541
 in vit A deficiency 93
 in vit B₁ deficiency 191
 in vit C deficiency 333
- F
- Factor I (vit B₁₂) 197
 Factor (pantothenic acid) 253
 Factor P 283
 Factor S (biotin) 469
 Factor T 5
 Factor U 55
 Factor V 99 34
 Factor W (biotin) 469
 Factor X (biotin) 469
 Factor X (vit E) 435
 Factor Y (vit B) 197
 2 Farnesyl 3 hydroxy 14 naphthoquinone vit K acty 497
 7 Farnesyl 14 naphthoquinone vit K acty 496
 2 Farnesyl 14 naphthoquinone oxide vit K acty 498
 Fat metabolism biotin relation 476
 choline relation 546
 essential fatty acid relation 538
 vit A relation 88
 vit B₁ relation 142
 vit B₂ relation 188
 vit B relation 213
 vit D relation 426
 vit E relation 457
 Fat soluble A 12 37 57
 Fatigue in vit B₁ deficiency 146
 Fatty acids essential 531
 Fermentation biotin relation 475
 vit B₁ relation 188
 Ferric chloride test for vit A 80
 for vit B 211
 Ferric chloride dipyritydyl test for vit E 452
 Fertility dose of vit E 45
 Fertility test for vit E 454
 Fertility vit 435
 Fibrin from fibrinogen 505
 Fibrinogen transformation into fibrin 505
 Fibrosis in vit E deficiency 464
 Filtrate factor (pantothenic acid) 253
 Flavin (riboflavin) 153
 Flavin-enzyme 171
 Flavin glucosides 167
 Flavin classification 154
 Fluorescence of dihydro-codehydrogenases II 235
 of lumichrome 160
 of riboflavin 157 179 182
 of thiochrome 120
 of vit A 89
 of vit A₂ 8
 of vit B₂ 157 179 182
 of vit K 485
 Fluorometric determination of dihydro-codehydrogenases I and II 233
 of dihydro-codehydrogenase II 237
 of vit B 18
 of vit B₁₂ in urine 147
 of vit B 18
 Folic acid 55
 Folin and Denis test for vit B₁₂ 210
 Folin test for vit C 319
 Foot odor in vit B₁ deficiency 146
 Formyldehydroazo-test for vit B 18
 Formamide in vit B synthesis 112
 Formate enzymatic dehydrogenation 227
 Formyl β ethoxy-ethyl propionate 107
 Formyl-ethyl propionate 109
 Formyl succinate 108 122
 Fortification of bread with vitamins 34 409
 Fortification of flour with vitamins 34
 Fortification of foods with vitamins 34 402
 Fortification of milk with vit D 403
 Fright due to essential amino-acid deficiency 541
 Fuchsin-sulfurous acid test for vit D 413

- l* Fuco ascorbic acid vit C acty 314
 Fumaric acid enzymatic reduction 177
 in citric acid cycle 137
 synthesis from oxaloacetic acid 137
 synthesis from pyruvic acid 137 138 139
 Fumaric hydrogenase 177
 Furfural test for vit C 321
 Furunculosis biotin therapy 477

G

- l* Galactonamide in *l* lyxose synthesis 309
l Galactonic acid growth factor 342
 in *l* lyxose synthesis 309
d Galactose in *l* lyxose synthesis 309
l Galactose in vit C synthesis 303 304
d Galacturonic acid in *l* lyxose synthesis 309
 Galacturonic acid in vit C biogenesis 312
 Gametogene is role of carotenoids in 87
 Gastrointestinal disorder in D hypervitaminosis 429
 in nicotinic acid defcy 246
 in vit A defcy 92
 in vit B₁ defcy 146
 in vit B₁ defcy 522
 in vit C defcy 334
 Gastrointestinal motility inositol relation 279
 nicotinic acid relation 245
 Geraniol enzymatic reduction 177
 2 Geranyl 14 naphthoquinone vit K acty 496
 Geronic acid from β -carotene 46
 from vit A 62
 Gestation in vit E defcy 454 455 459 461
 Gibbs phenol indophenol test for vit B₄ 210
 Gingivitis in vit C defcy 333
 in vit M defcy 525
 Giri test for vit C 390
 Glossitis in nicotinic acid defcy 246
 in riboflavin defcy 191
 Glucide X 290
l Gluco ascorbic acid vit C acty 314
d Gluco hepto ascorbic acid vit C acty 314
 Gluconic acid from glucose 247
 growth factor 542
 Glucose enzymatic oxidn 174 177 227
 hyd n to *d* sorbitol 304
 in *l* xylose synthesis 308
 in vit C synthesis 304
 Glucose oxidase 171
 Glucose 6 phosphate enzymatic dehyd n 227
 Glucuronic acid growth factor 542
 in vit C biogenesis 312
 Glutamic acid enzymatic dehyd n 227
 Glutathione vitagen nature 549
 Glycerin aldehyde phosphate enzymatic dehyd n 174
 Glycerin phosphoric acid enzymatic dehyd n 174
 Glycerol diethyl ether as solvent for vit D 403
 α Glycerophosphate enzymatic dehyd n 227
 Glycine vit B₄ action 592
 Glycogen metabolism vit B₁ relation 149
 Glycogenotropic hormone relation to vit A 91
 Glycolysis vit B₁ relation 188
 Glyoxalate ethyl in vit C synthesis 310
 Gold trichloride test for vit C 321

for vit E 453

- Grass juice factor 598
 Guanine in growth factor for lactobacillus 596
 Guinea pig test for vit C 3 3
 for vit P 516
l Gulonic acid in *l* gulose synthesis 305
 oxidn to 2 keto gulon c acid 307
l Gulonic acid lactone in *l* xylose synthesis 308
l Gulo c in vit C synthesis 303 304 305
 relation to vit C 299
 synthesis 305
 Gum arabic growth factor 549
 Gums ulceration in vit M defcy 525
 Gurwitsch rays for activation of provit D 404
 Guvacin in nicotinic acid biogenesis 226

H

- Head retraction in vit B₁ defcy 146
 Heart failure in vit B₁ defcy 146
 Hematoma in vit C defcy 333
 Hemeralopia in vit A defcy 92
 Hemochromogen oxidn vit C participation 328
 Hemoglobin oxidn vit C participation 328
 Hemophilia relation to vit K defcy 507
 Hemorrhages in A hypervitaminosis 95
 in essential methyl group defcy 544
 in vit C defcy 330 333
 in vit K defcy 507
 in vit P defcy 518
 Hepato flavin 153 156
 Hexantheate 72
 2 β Heptenyl 3 hydroxy 14 naphthoquinone vit K acty 497
 Hesperidin 513-518
 Hesperitin 513-518
 Hetero-vit B₁ 127
 2 n Hexadecyl 14 naphthoquinone vit K acty 496
 β 7 5 6 7 8 Hexahydro vit K₁ vit K acty 497
 Hexose diphosphate 125
 Hexose monophosphate enzymatic dehyd n 174
 Hexuronic acid (vit C) 289
 Hexuronic acid in vit C synthesis 303
 Histamine enzymatic oxidn 177
 Histidine essential amino-acid 340
 Hock disease in vit B₁ defcy 523
 Holoenzymes classification 227
 terminology 227
 Hormones defn 3
 Hormoxyme 14
 Hydroquinone as antioxidant for vit A 68
 Hydroquinone poisoning effect of p amino benzo c acid 285
 Hydroquinone diacetate vit K acty 497
 β Hydroxy butyric acid enzymatic dehyd n 227
 α Hydroxy γ butyrolactone pantothenic acid acty of condensation product with β alanine 205
 3 Hydroxy β β carotene 41 46
 7 Hydro y-cholesterol in 7 dehydro-cholesterol synthesis 360 361
 β 7 Hydro y-cholesterol 360 361
 7 Hydroxy cholesterol 3 benzoate in 7-dehydro-cholesterol synthesis 360

- 7 Hydroxy-cholesterol-dibenzoate in 7 dehydro-cholesterol synthesis 360 361
- 3 Hydroxy 4,5 di (hydroxy methyl) 2
methyl pyridine 197
- 2 Hydroxy 3 d methyl allyl 14 naphthoquinone, vit K acty 49"
- a Hydroxy β d methyl γ butyrolactone synthesis 259 261
- 2 Hydroxy 4 methyl δ acetoxy ethyl thiazol synthesis 110 113
- a Hydroxy α methyl γ butyrolactone pantothenic acid acty of condensation product with β -alanine 265
- a Hydroxy β d methyl γ butyrolactone pantothenic acid acty of condensation product with β -alanine 265
- 2 Hydroxy 14-naphthoquinone vit K acty 497
- a Hydroxy 14 naphthoquinone vit K acty 497
- Hydroxypantothenic acid 264
- Hydroxy pyridine 202
- a Hydroxy δ valerolactone pantothenic acid acty of condensation product with β -alanine 265
- a Hydroxy γ valerolactone pantothenic acid acty of condensation product with β -alanine 265
- Hyperketoatosis in vit A deficiency 93
- Hypervitaminosis 32
- Hypoglycemia vit C therapy 334
- Hypoplastic tooth in vit A deficiency 93
- Hypovitaminosis 31
- Hypoxanthine enzymatic oxidation 175
- I
- Idonic acid from vit C 297
- Ildose in vit C synthesis 303 304
- β -Immunazoly-alanine 540
- Imino-glutarate catalytic hydride 229
- Indicator yellow 90
- β Indolyl-alanine (tryptophane) 540
- Indoxyl-ethylamine excretion in nicotinic acid deficiency 245
- Infections in vit A deficiency 92
- in vit C deficiency 333
- in vit D deficiency 423
- in vit E deficiency 463
- Inositol 275
- Inositol 275
- d hydrate 277
- esters inositol acty 278
- hexa-acetate 276
- hexaphosphate 276
- solubility 277
- Insomnia in vit B deficiency 214
- Insulin relation to vit B₁ 145
- relation to vit B 190
- Intercellular substances influence of vit C 309
- International Unit of riboflavin 185
- of vit B 83
- of vit B 13
- of vit B₁ 185
- of vit C 323
- of vit D 416 417
- of vit E 455
- Intoxication vit B effect 146
- vit. C effect, 330
- vit P effect 517
- Intradermal test for vit C deficiency 330
- Intrinsic factor in pernicious anemia 507
- Iodides vit D acty 409
- Iodine test for vit C 316
- Iodine vit D acty 409
- α Ionone 46
- β Ionone 46 63
- β Ionylidene-ethyl acetate 63 65
- Irradiation of provit D 368
- Irradiation sickness nicotinic acid treatment 247
- Irritability in vit B₁ deficiency 214
- Iso-alloxantine 160
- Iso-ascorbic acid vit C acty 314
- Iso-dehydro-cholesterol 361
- Irradiation 300
- Iso-geron c acid 46
- Isoleucine essential amino-acid 540
- Isonitric acid essential fatty acid acty 336
- Iso-prene rule in provit A structure 50
- in vit E structure 442
- Iso-pseudo-cumenol from α -tocopherol 441
- Iso-pyro-calciferol from vit D₂ 390 397
- J
- Jansen test for vit B 128
- Jaundice absorption of provit A 84
- of vit A 84
- vit K therapy 500
- Juglone vit K acty 497
- K
- Keratitis interstitial in riboflavin deficiency 190
- Keratomalacia in vit A deficiency 190
- Keratinosis in vit A deficiency 92
- a Keto-carboxylic acid decarboxylation 136
- a Keto-butyric acid decarboxylation 136
- a Keto-glutaric acid decarboxylation 136
- in citric acid cycle 137
- nicotinic acid enzymes relation 227 229
- synthesis from citric acid 137 227
- synthesis from oxaloacetic acid 137
- synthesis from pyruvic acid 137 138
- 3 Keto-4 gulo-furano-lactone 289
- 2 Keto-1 gulo-nate methyl vit C acty 314
- vit C acty 314
- Keto-hexonic acid in vit C synthesis 303
- 3 Keto-hexonic acid in vit C synthesis 303
- 5-Keto-hexuronic acid in vit C synthesis 303
- 13 Keto-threo-hexuronic acid in vit C synthesis 303
- a Keto-valeric acid decarboxylation 136
- Kidney tones in vit A deficiency 92
- Kinnear's and Peters test for vit B₁ 128
- Koagulations vit 481
- L
- Lactation in β -amino-benzoic acid deficiency 286
- essential fatty acid deficiency 339
- in essential methyl group deficiency 543
- Lactation vit 524
- Lactic acid enzymatic dehydrogenation 174 200
- synthesis from pyruvic acid 139 140

- Lactobacillus growth factor 576
 Lactobacillus test for biotin 474
 for nicotinic acid 249
 for riboflavin 184
 Lactochrome 154
 Lactoflavin 153 156
 Lamenesis in vit B₁ deficiency 140
 Langenbeck cycle 144
 Lapachol vit K acty 497
 Laquer Unit of vit D 417
 Lassitude in biotin deficiency 477
 in vit B₁ deficiency 146
 Lawsone vit K acty 497
 Lead poisoning vit C therapy 330 334
 Leprotene constitution 42 48
 isolation 45
 properties 42
 Lethal dose of nicotinic acid 249
 of vit B₁ 150
 of vit B₂ 215
 Leucine essential amino acids 540
 Leuco-lactoflavin 160
 Leuco riboflavin 160 180
 Levulin aldehyde from vit K₂ 480
 Levulinic acid 51
 Liebermann Burchard test for provit D 367
 Light filters for irradiation of provit D 369
 Line test in vit D assays 410
 Linoleic acid 531
 constitution 534
 essential fatty acids acty 536
 isolation 533
 occurrence 532
 properties 534
 separation from linolenic acid 533
 Linolenic acid 531
 constitution 534
 isolation 533
 occurrence 532
 properties 534
 separation from linoleic acid 533
 Linolic acid essential fatty acid acty 536
 Lipocic 476
 Lipochromes classification 154
 Lipotropic factor choline action 544
 inositol action 279
 Liver cirrhosis in essential methyl group deficiency 544
 Liver damage in panthothenic acid deficiency 269
 Liver filtrate factor 253
 Lovibond Unit of vit A 78 83
 Lovibond Unit (Wolff) of vit A 83
 Lumichrome 160 179
 synthesis 163
 Lumiflavin 158 159 179
 synthesis 163
 Lumilactoflavin 158
 Lumilactoflavin test for vit B₂ 183
 Lumisterol 370 375
 Lutein 52
 β lycopene 41 46
 lycopene 46 48
 Lychromes classification 153 154
 Lysine essential amino acid 540
 Lyxo hexonic acid in vit C synthesis 303
 304
 Lysine in vit C synthesis 308
 synthesis 309
- Magnesium metabolism relation to riboflavin 190
 Maleic acid enzymatic reduction 17
 Maleic acid enzymatic dehydro 174 177
 in citric acid cycle 137
 synthesis from oxaloacetic acid 137
 synthesis from pyruvic acid 137 138 139
 Mallory stain 90
 Malonic aldehyde carboxylic acid from linolenic acid 534
 Manganese in vit C biogenesis 312
 Manganese metabolism carboxylase relation 146
 vit B₁ relation 145
 Mannose in vit C biogenesis 312
 Melanin formation β amino-benzoic acid effect 285
 Melophanic acid from neoergosterol 307 308
 Mental disorder in nicotinic acid deficiency 246
 Mercuric chloride test for vit C 371
 Meso inositol 275
 Mesoxylate ethyl in vit C synthesis 311
 Methionine 543
 essential amino acid 540
 properties 544
 synthesis by transmethylation 547
 4 Methyl 5 β acetoxy ethyl thiazole synthesis 113
 2 Methyl 2 alkoxy 3 chloro tetrahydrofuran 117
 Methyl β amino aceto phenone in test for vit B 19
 2 Methyl 4 amino 5 amino methyl pyrimidine 116
 synthesis 100 106 108
 2 Methyl 4 amino 5 bromo methyl pyrimidine 116
 synthesis 108 107 108
 2 Methyl 4 amino 6 chloro pyrimidine 5 ethyl acetate synthesis 108
 2 Methyl 4 amino 5 cyano pyrimidine synthesis 106
 2 Methyl 3 amino 4 ethoxy methyl 5 amino methyl pyridine 204
 2 Methyl 3 amino 4 ethoxy methyl 5 cyano 6 chloro pyridine 204
 2 Methyl 4 amino 5 ethoxy methyl pyrimidine synthesis 107
 2 Methyl 3 amino 4 hydroxy methyl 5 amino methyl pyridine 204
 2 Methyl 4 amino 5 hydroxy methyl pyrimidine 116
 synthesis 108
 2 Methyl 4 amino 6 oxy pyrimidine 5 ethyl acetate synthesis 108
 2 Methyl 4 amino pyrimidine 5 acetamide synthesis 108 109
 2 Methyl 4 amino pyrimidine 5 ethyl acetate synthesis 108
 2 Methyl 4 amino 5 thioformamido-methyl pyrimidine synthesis 116
 Methylation by choline 547
 a Methyl β benzoyl propionic acid in 2 methyl 14 naphthoquinone synthesis 491
 2 Methyl 3 benzyl 14 naphthoquinone vit K acty 496
 2 methyl 3 carbethoxy 14 naphthohydroquinone vit K acty 497

- 2 Methyl 4 chloro 5 ethoxy methyl pyrimidine synthesis 107
Methyl α chloro γ ethoxy propyl ketone 111
Methyl α chloro γ hydroxy propyl ketone 122
2 Methyl 4 chloro pyrimidine 5 ethyl acetate synthesis 108
2 Methyl 3 cinnamyl 14 naphthoquinone vit K acty 496
2 Methyl 3 cinnamyl 14 naphthoquinone oxide vit K acty 498
2 Methyl coumaran vit E acty 451
 β Methyl crotonaldehyde 51
 γ Methyl cyclopenteno phenanthrene 352
S-Methyl cytosine as essential methyl group supplier 545
2 Methyl 14 diacetoxynaphthalene 3 acetaldehyde from d hydro-vit K₁ 488 from vit K₁ 489
2 Methyl 14 diacetoxynaphthalene 3 acetic acid from d hydro-vit K₁ 487
2 Methyl 3 difarnesyl 14 naphthoquinone (vit K) 490
2 Methyl 3 difarnesyl 14 naphthoquinone vit K acty 496
2 Methyl 5,8-dihydro 14 naphthohydroquinone vit K acty 499
2 Methyl 3 (β γ dihydrophytyl) 14 naphthoquinone vit K acty 496
Methylene blue test for vit C 319
2 Methyl 3 farnesyl 14 naphthoquinone vit K acty 496
2 Methyl 3 geranyl 14 naphthoquinone vit K acty 496
Methyl glyoxal occurrence in vit B deficiency 142
Methyl group essential 543
2 Methyl 3 hydrocinnamyl 14 naphthoquinone vit K acty 496
2 Methyl 4 hydroxy 5 amino methyl pyrimidine synthesis 122
2 Methyl 4 hydroxy 5 chloro methyl pyrimidine synthesis 122
2 Methyl 3 (γ hydroxy dihydrophytyl) 14 naphthoquinone vit K acty 497
2 Methyl 4 hydroxy 5 ethoxy methyl pyrimidine synthesis 107 109
4 Methyl 5 β hydroxy ethyl thiole 111 116 119 synthesis 111 112 113
2 Methyl 4 hydroxy 5 hydroxy methyl pyrimidine synthesis 122
2 Methyl 3 hydroxy isocrotyl 20
2 Methyl 3 hydroxy 14 naphthoquinone vit K acty 497
2 Methyl 5 hydroxy 14 naphthoquinone vit K acty 497
2 Methyl 4 hydroxy pyrimidine 5 ethyl acetate synthesis 108 122
- Methyl 4 hydroxy pyrimidine 5 methyl sulfonic acid 105 synthesis 109
Methyl inositol inositol acty 278
Methyl opoalacet aldehyde from ergosterol 353 354 from 1-methyl 374 from vit D₁ 393
2 Methyl 3-methoxy soquinoline in vit B synthesis 206
2 Methyl 3 methoxy pyrimidine 4,5-dicarboxylic acid 206
2 Methyl naphthalene oxide 490 vit K acty 500
2 Methyl 14 naphthohydroquinone toxicity 509 vit K acty 500
2 Methyl 14 naphthohydroquinone diacetate vit K acty 498
2 Methyl 14 naphthohydroquinone dibenzoyl ether vit K acty 498
2 Methyl 14 naphthohydroquinone dibenzyl ether vit K acty 498
2 Methyl 14 naphthohydroquinone dimethyl ether vit K acty 498
2 Methyl 14 naphthohydroquinone monoethyl ether vit K acty 498
2 Methyl 14 naphthohydroquinone sodium di-phosphate 494 vit K acty 498
2 Methyl 14 naphthohydroquinone sodium di-sulfate vit K acty 498
1 Methyl- γ naphthol vit K acty 500
2 Methyl 1 naphthol vit K acty 500
3 Methyl 1 naphthol vit K acty 500
3 Methyl 2 naphthol vit K acty 500
4 Methyl 1 naphthol vit K acty 500
2 Methyl 14 naphthoquinone 481 properties 485 solubility 485 synthesis 491 toxicity 509 vit K acty 496
2 Methyl 14 naphthoquinone 3 acetic acid from vit K₁ 487
2 Methyl 14 naphthoquinone oxide vit K acty 498
2 Methyl 14 naphthoquinone 3 sodium sulfonate 493
2 Methyl 1 naphthylamine vit K acty 499
2 Methyl 3 nitro 4 ethoxy methyl 6 cyano 6 chloro pyridine 204
2 Methyl-3-octadecyl 14 naphthoquinone vit K acty 496
2 (δ Methyl γ pentenyl) 14 dihydro anthraquinone vit K acty 497
9 Methyl permaphthenone 7 vit K acty 498
 α Methyl γ phenyl butyric acid in 2-methyl 14 naphthoquinone synthesis 491
2 Methyl 2 phytyl 3 dihydro 14 naphthoquinone vit K acty 498
2 Methyl-3-phytyl 14 naphthoquinone (vit K₁) 484 vit F acty 496
6 Methyl 9 (δ 1 ribtyl) isoalloxazine 181
7 Methyl 9 (δ 1 ribtyl) isoalloxazine 181
4 Methyl thiazole 5 carboxylic acid 110 synthesis 110 111
Methyl testosterone 20
2 Methyl 1,2,3,4-tetrahydro-naphthalene in 2-methyl 14 naphthoquinone synthesis 491
2 Methyl 5,6,7,8-tetrahydro-14 naphthoquinone vit K acty 499
2 Methyl 5,6,9,10-tetrahydro 14 naphthoquinone vit F acty 499

- 2 Methyl 1 tetralone in 2 methyl 1 4 naphthoquinone synthesis 491
vit K acty 500
- 3 Methyl 1 tetralone vit K acty 500
- Methyl tocol vit E acty 449
- 2 Methyl 3 (β γ trimethyl allyl) 1 4 naphthoquinone vit K acty 496
- Methyl vinyl ketone 51
- Microbiological method for the detm of vitamins 23
- Milk irradiation 371 403
- Milk fever vit D therapy 493
- Mineral metabolism in vit D defcy 420
- Mineral metabolism test for vit D defcy 429
- Mineral oils use as solvent for vit A 84
- Mitogenetic radiation for activation of provit D 371 404
- Mold growth test for vit B₁ 131
- Möller Barlow's disease in vit C defcy 333
- Molybdenum phosphotungstic acid test for vit A 80
for vit C 320
- Moore blue unit 78
- Mouse factor 527
- M R C Unit of vit D 416 417
- Muscle pains in biotin defcy 477
- Muscular dystrophy in vit B₆ defcy 214
in vit E defcy 459 461 463 464
- Muscular weakness in vit B₁ defcy 146
in vit B₆ defcy 522
in vit D defcy 428
- Mussel provit D constitution 359
properties 349
- Myasthenia in vit B₁ defcy 215
- Myelin changes in riboflavin defcy 191
- Myelopathy vit E therapy 464
- Myopia in vit A defcy 92
- Mytilitol inositol acty 278
- Myxomanthia constitution 42 48
isolation 45
occurrence 39
properties 42
- N**
- Nasman test for vit B₁ 129
- β Naphthoic acid in 2 methyl 1 4 naphthoquinone synthesis 491
- β Naphthoic acid nitrile in 2 methyl 1 4 naphthoquinone synthesis 491
- 1 Naphthol vit K acty 500
- β Naphthoquinoline in nicotinic acid synthesis 224
synthesis 223
- Naphtho-tocopherol vit E acty 450
vit K acty 450 493
- β Naphthylamine in nicotinic acid synthesis 224
- Necrosis (kidney) in essential methyl group defcy 544
- Necrosis (muscle fibers) in vit E defcy 462
- Neorgosterol 357 358
- Neo tocopherol 436
- Nephritis vit P therapy 513
- Nerve excitement vit B₁ relation 142
- Nervous system in biotin defcy 477
in nicotinic acid defcy 245 246
in valine defcy 541
in vit B₁ defcy 142 146
in vit B₆ defcy 214
in vit E defcy 459 462
- Neuralgia in vit B₁ defcy 146
- Neurological lesions in vit A defcy 92
- Neuromalacia in riboflavin defcy 191
- Niacin 219
- Niacin amide 219
- Niamife 219
- Nicotinamide ⁹¹⁹
absorption spectrum ²⁹²
N diethyl 239
glucosido iodide nicotinamide acty ⁹³⁹
iodomethylate 238
N methyl 239
separation from coenzymes 222
separation from nicotinic acid 221 242
- Nicotinate ethyl 239
- Nicotine oxidn to nicotinic acid 223 224 225
- Nicotinic acid 219
absorption spectrum 222
amidation 225 244
decarboxylation 223
separation from nicotinamide 221 24⁹
vit B₆ acty 522
- Nicotinuric acid nicotinic acid acty ⁹³⁹
- Night blindness in vit A defcy ⁹⁷ 93
- Nitric acid test for vit E 453
- Nitro brucine test for vit C 322
- Nor allo cholanolic acid 350 351
- Nor allo-cholanolic acid 3 acetoxy 352
- Nuclease activation by vit C 328
- Nyctalopia in vit A defcy ⁹⁷
- O**
- 2 n Octadecyl 1 4 naphthoquinone vit K acty 496
- Octa hydro vit D₃ 392
- Oestrus cycle test for essential fatty acids 537
- Oil for vit A preparations effect on vit utilisat ion 84
- Oils as solvents for vit A 58
- Ophthalmalin 57
- Opisthotonus in vit B₁ defcy 146
- Ornithine in nicotinic acid biogenesis 2 6
- Oryzamin 99
- Osazone furfural test for vit C 3⁹¹
- Oslo Unit of vit D 417
- Osteodentine in vit C defcy 333
- Osteomalacia in vit D defcy 428
- Osteoporosis in vit D defcy 428
- Ovary atrophy in vit A defcy 92
- Ovodavin 153 156
- Oxalic acid test for vit C 322
- Oxaloacetic acid enzymatic hydri 227 ⁹²⁹
in citric acid cycle 137
synthesis from pyruvic acid 136
- 22 22 Oxido ergosterol properties 349
synthesis 364
- 7 Oxo cholesterol acetate in 7 dehydro-cholesterol synthesis 360 361
- 7 Oxo cholesterol benzoate in 7-dehydro-cholesterol synthesis 360
- Oxy β -carotene preparation 75
vit A acty 75
- P**
- Pacini Linn Unit of vit E 455
- Pancreas fibrosis vit A defcy due to 93

- Pantothen 253
 Pantothenic acid 253
 protein-complex 255
 vit B₅ acty 521
 Papain activation by vit C 328
 Para amino benzo c acid 283
 Parathema in vit B₁ defcy 148
 Paralysis in argin ne defcy 541
 in essential methyl group defcy 545
 in glycine defcy 41
 in riboflavin defcy 191
 in vit B₁ defcy 146
 in vit B₁ defcy 521
 in vit E defcy 462
 Parathyroid vit D relation 426
 Paravitaminosis defo 32
 Pellagra in nicot nic acid defcy 246
 in pantothenic acid defcy 268
 in riboflavin defcy 19²
 in vit B₁ defcy 214
 vit B₁ therapy 523
 multiple vit defcy 246
 preventive factor 219
 use of term 253
 Pell gr mine 219
 Pemphigus riboflavin treatment 192
 2 2 5 7 8 Pentamethyl 6 hydroxy chroman vit B
 acty 451
 2 3 4 6 7 Pentamethyl 5 hydroxy coumaran vit
 E acty 451
 Perhydro-vit A 63
 vit A acty 75
 Peristalsis in meso tol defcy 279
 in nicotinic acid defcy 245
 Periwinkle provit D constitution 359
 properties 349
 Permeability vit (vit P) 513
 Perosis in choline defcy 545
 in vit B₁ defcy 53
 Pharmacologic l action of 2 methyl 1 4 naphtho
 quinone 509
 of nicotamide 249
 of nicotinic acid 249
 of pantothen c acid 270
 of provit A 95
 of vit A 95
 of vit B₁ 149
 of vit B 193
 of vit B 215
 of vit C 337
 of vit D 479
 of vit E 464
 of vit K 508
 Phenylalanine essential amino acid 540
 Phenyl-crotyl alcohol enzymatic reduction 177
 o-Phenylene-diamine 163
 3 Phenyl pyridine 2 4
 Pheron 171
 Phlorone vit K acty 493
 Photo t ve l i 179
 activation by vit C 379
 in vit D defcy 473
 Phosphate dono s 12
 6 Phosphogluconate nicotinamide enzymes rela
 tion 227
 Phospho-ly taldehyde enzymatic dehyd 2 7
 Phosphoglycer c d 125
 from a glycerophosphate 2 7
 Phosphomolybd c a c d t e t for vit C 3 0
 Phosphopyruvic acid 125
 Phosphore ten e of glow wo m 189
 Phosphoric acid colo reaction for vit A 80
 Phosphorus absorption in vit D defcy 42
 Phosphorus m tabolism in vit D defcy 470
 42² 424 4 5 429
 Phosphorus pentachloride t e t for vit D 413
 Phosphorylation of riboflavin 177-179 186
 of vit B₁ 174 135
 Photoflavin 1 8
 Photophobia in riboflavin defcy 192
 Photosensitizer in irradiation of provit D 370
 Phtalic acid from vit F₁ 487
 Phtalimide in vit B synthesis 207
 α Phtalimide propionic acid ester .07
 Phtocol in vit K₁ synthesis 493
 occurrence 482
 vit K acty 497
 Phylloquinone α β etc 481
 Phytal methods for the detn of vits 21 2
 Phytadene in vit F synthesis 445
 Phytic acid occurrence 276
 Phytol in vit E synthesis 445
 in vit K₁ synthesis 49
 oxidn 488
 Phytol halide in vit E synthesis 445
 2 Phytol 1 4 naphthoquinone vit K acty
 496
 2 Phytol 1 4 naphthoquinone oxide vit K acty
 499
 Phytin ino tol acty 278
 Phytostathins α
 β Picoline in nicot n c acid synthesis 274
 Picolin c a c d d ca bo yl t n 3
 Pigmentation p amino benzo c acid effect 285
 pantothenic acid effect 69
 Pilot dye 61
 Pitarelli's test for vit C 371
 Pituitary anterior lobe vit D relation 4 7
 vit E relation 459 460
 Pneumonia nicotinic acid therapy 247
 vit J therapy 523
 Polarograph c determination of riboflavin 183
 of vit C 318
 Polyenes 46
 Polyn synthesis 65 66
 Polyneurmin 99
 Polyneuritis in vit B₁ defcy 146
 Polyterpenes building principle .0
 Porphyropsin 90
 Porphyrimuria in K hypervitaminosis 509
 Porphyrimuria: nicotin acid defcy 246 248
 Poulsson Unit of vit D 417
 P P factor 219
 Pr bluda and McCollm test for vit B 1 9
 148
 Precord al d stre s in biotin defcy 477
 P escorbatic state det tion 335
 Proline in nicotin acid bog nes s 6
 Pronosol 70
 Prophylactic Unit of vit D 417
 Prop on ldehyde from l nolic acid 534
 Propyl ne glycol at solv nt for vit D 403
 2 n Propyl 1 4 naphthoquinone vit K acty
 496
 Prosthetic group of enzyme 171 277
 Pro tachysterol 3 3 377

- Thiamin 99
 (see also under Vit B₁)
 Thiazole 5 carboxylic acid nicotinic acid acty
 240
 Thiochrome 120
 absorption spectrum 120
 fluorescence 121
 isolation 120
 occurrence 120
 properties 120
 synthesis 121 122
 vit B₁ acty 126
 Thiochrome test for vit B₁ 128 147
 γ Thiocyan γ aceto propyl acetate synthesis
 112
 Thioformamide in vit B₁ synthe is 111 112
 Thiourea in vit B₁ synthesis 122
 l Threo 3 keto hexuronic acid lactone 289
 l Threonine acid from vit C 298
 Threonine essential amino acid 340
 l Threo 2 3 4 5 6 pentoxy hexen 2 carboxylic acid
 lactone 289
 l Threonine in vit C synthesis 310
 Thrombin in blood coagulation 505
 Thrombocytosis in factor T defcy 525
 Thrombokinas in blood coagulation 505
 Thrombopenia relation to vit K defcy 507
 Thromboplastin in blood coagulation 505
 Thymus in essential methyl group def y 544
 in pantothenic acid defcy 269
 in riboflavin defcy 190
 in vit D defcy 427
 in vit E defcy 462
 Thyroid in vit E defcy 459 460
 Thyroxine relation to vit A 91
 relation to vit B₁ 144
 relation to vit B 190
 relation to vit C 332
 Tissue saturation test for vit B₁ 193
 Titanous chloride test for vit K 501
 Tocol defn 435 (footnote 5)
 vit E acty 449
 Tocopherol 435
 nomenclature 436 (footnote 7)
 α Tocopherol 435
 constitution 440
 separation from β tocopherol 438 439
 vit E acty 449
 dl α Tocopherol vit E acty 449
 β Tocopherol 436
 constitution 443
 separation from α tocopherol 438 439
 vit E acty 449
 γ Tocopherol 436
 constitution 444
 vit E acty 449
 Tocopherol acetate vit E acty 449
 Tocopherol alloph nate vit E acty 449
 Tocopherol esters vit E acty 449
 Tocopherol ethers vit E acty 449
 Tocopherol prop onate vit E acty 449
 Tocopherol quinones vit E acty 449
 α Tocopherolquinone vit K acty 438
 dl α Tocopherol phosphoric acid ester vit E
 acty 449
 Tocopheryl quinol 442
 Toe curled in riboflavin defcy 191
 Toluene 2 3 4 5 tetra-carboxylic acid 356 357
 o Toluidine magnesium iodide 63
 Tooth in vit A defcy 93
 in vit C defcy 323
 in vit D defcy 427 428
 Tortelli Jaffe test for provit D 367
 Tortelli Jaffe test for vit D 413
 Torula 99
 Toxisterol 380
 Transient orange 90
 Transmethylation 547
 Trichloro acetic acid test for provit D
 for vit A 80
 Trichochromogenic factor 284
 Trigonelline nicotinic acid acty 239
 1 2 4 Trihydroxy anthraquinone vit
 497
 9 10 12 Trihydroxy stearic acid essen
 acid acty 536
 2 3 5 Trimethyl 1 4 benzoquinone vit
 498
 2 7 Trimethyl-coumaran vit E acty
 67
 Trimethylene 9 (11 arabityl)
 azine 181
 Trimethyl ethyl hydroquinone vit E
 Trimethyl hydroquinone from β tocoph
 from γ tocopherol 444
 in α tocopherol synthesis 445
 vit E acty 450
 2 4 5 Trimethyl 3 hydroxy pyridine 205
 2 6 10 Trimethyl pentadecanone 14 from
 488
 2 3 5 Trimethyl 6 phytyl 1 4 benzoquin
 K acty 498
 5 7 8 Trimethyl tocol 435)
 Triose catabolism nicotinamide enzyr
 tion 227
 Triphosphopyridine nucleotide 234
 Tryptophane catabolism in nicotinic ac
 240
 essential amino acid 540
 Tschugajeff reaction for provit D 368
 Tuberculosis in vit C defcy 333
 Turkey Unit of vitamin D defn 410
 l Tyrosine poisoning vit C effect 330
 Tyrosinase p amino benzoic acid effect
 vit C effect 328

U

- Ulcers vit C therapy 334
 vit M therapy 525
 Ultraviolet light activation of provit D
 Uracil d riboside removal from pantothe
 246
 Uranyl acetate test for vit C 320
 Urea c activation by vit C 3 8
 Uric acid 170
 Uridine removal from pantothenic acid :
 Uridy calculi in vit A defcy 92
 Urinary excretion of p amino benzoic acid
 of creatine in vit E defcy 404
 of nicotinic acid 241 247
 of nicotinuric acid 244 247
 of pantothenic acid 268 70
 of porphyrin 246 248 219
 of riboflavin 187
 of trigonelline 244 247
 of vit B₁ 135 147

- of vit B 212 215
- of vit C 3 5 3 8 333 335
- of vit E 456
- Uroflavin 187
- U S Pharmacopoeia Reference Cod Liver Oil 416
- U S Pharmacopoeia Unit of vit A 83
 - of vit B₁ 13
 - of vit C 313
 - of vit D 417 417
- Urine index in vit E defn 454
- V
- Vaginitis senile in vit A defcy 92
- Valine sentalamino acid 540
- Vanadium test for vit C 31
- Vascular lesions in vit A defcy 93
- Vascular permeability in vit P defcy 518
- Verdoflavin 161
- Verdo hemochromogen from hemoglobin 508
- Vincent's infection and nicotinic acid defcy 247
- Vioferol 341
- Vision influence of vit A 89 90 93
 - influence of vit B 189 191
 - influence of vit C 333
- Vitagens 529
 - classification 6
 - defn 6
 - descript n 531
- Vitamin defn 3
- Vit A blood level 93
 - classification 4 38
 - defn 4
 - esters 62
 - vit A acty 75
 - homologs 70 71
 - fluorescence 82
 - influence on carbohydrate metabolism 88 ✓
 - influence on cholesterol metabolism 88
 - influence on fat metabolism 88
 - in plants 83
 - mobilization 89
 - protein complex 89
 - storage 86
 - vit E influence 460
- Vit A 57
- Vit A₂ fluorescence 8
 - in mammalian nutrition 71
 - physiological significance 71
- Vit B complex 13 15
- Vit B absorption spectrum 103
 - defn in presence of vit B₁ pyrophosphate 130
 - effect on plants 133
 - 2 ethyl homolog 17
 - in blood serum 135
 - phosphorylation 14
 - salts 103
 - separation from riboflavin 101 102
 - sensitivity 103
 - synthesis 116
 - transformation 113
- Vit B₁ active principle excitement 142
- Vit B bromide hydrobromide conversion into the chloride hydrochloride 116
- Vit B-deficiency 126 143
- Vit B₁ monophosphate 124 125 13
 - occurrence 101
 - vit B₁ acty 16
- B₂ phosphorus protein complex 101
- Vit B₁ protein complex 101 135
- Vit B pyrophosphate 122
- occurrence 101
- Vit B₂ (5 under Riboflavin)
- Vit B₂ (abandoned name for pantothenic acid) 53
- Vit B₂ 51
 - relation to pantothenic acid 2 3
- Vit B 51
 - vit B₂ 52
 - relation to nicotinic acid 219
- Vit B₂ absorption spectrum 200
 - blood level 219
 - solubility 200
 - vit B acty 219 222
- Vit B esters vit B₂ acty 209
- Vit B ethers vit B₂ acty 209
- Vit B protein complex 198 199
- Vit B₂ 522
- Vit B₂ (adenylic acid) 5 2
- Vit B 5 3
- Vit B₂ 53
- Vit B₂ (biotin) 469
- Vit B (pantothenic acid) 83
- Vit B (pantothenic acid) 253
- Vit C 289
 - absorption spectrum 294 295 297 315
 - dissociation constants 295
 - optical activity 295
 - oxidation reduction potential 295
 - solubility 295
- Vit C₁ 5 3
- Vit D 341 383
 - constitution 401
 - isolation of natural vit D 387
 - isolation of synthetic vit D 388
- Vit D milk 403
- Vit D₁ 383 389
 - constitution 391
 - isolation 375
 - nomenclature 374
 - preparation 375
- Vit D₂ 341 373 83
 - addition compounds 389
 - constitution 390
 - properties 389 416
 - stability 389
- Vit D 341 383
 - constitution 399
 - properties 390
- Vit D₃ 341 383
 - constitution 400
 - properties 390
- Vit D 341 383
 - constitution 401
- Vit D₄(?) 383 388
 - constitution 401
- Vit E 430
 - antioxidative properties 440
 - antioxidant for vit A 68
 - relation to stored vit A 460
 - solubility 440
- Vit F (fatty acid) 531
- Vit F (histo calin methyl vit B₁) 99
- Vit F (unsaturated fatty acids) 531
- Vit. G (abandoned name for pantothenic acid) 53

Vit G (historical name for riboflavin) 153
 Vit H (biotin) 46J
 Vit H (historical name for vit B₆) 197
 Vit H (historical name for a trout growth factor) 197
 Vit I 5⁹²
 Vit J 523
 Vit K 481
 water soluble forms 493
 Vit K₁ 481
 absorption spectrum 485
 fluorescence 485
 vit K acty 496
 Vit K₂ 481
 vit K acty 496
 Vit K deficiency by chloroform intoxication 503
 by *p* toluene diamine intoxication 503
 Vit K₁ hydroquinone diacetate vit K acty 498
 Vit K₁ hydroquinone diposphoric acid vit K acty 498
 Vit K₁ hydroquinone potassium disulfate vit K acty 498
 Vit K₁ oxide vit K acty 498
 Vit L₁ 5⁹⁴
 Vit L₂ 524
 Vit M 524
 Vit P 513
 Vitazyme 5 14

W

Warburg's Coferment 234
 Water imbibition in vit B₁ synthesis 14⁹
 Water metabolism nicotinic acid relation 245
 vit B₁ relation 142
 Water soluble B 12
 Wound healing in vit C deficiency 333

X

Xanthine enzymatic oxidn 175

Xanthine oxidase 171
 Xerophthalmia in vit A deficiency 92
 Xanthophyll 52
 X ray sickness nicotinic acid treatment 247
 X rays for activation of provit D 371
o Xylene in riboflavin synthesis 170
p Xylenol from β tocopherol 443
 3,4 Xyldene in riboflavin synthesis 165
l Xylo ascorbic acid 289
o Xylo hydroquinone in γ tocopherol synthesis 445
 vit E acty 450
m Xylo hydroquinone vit E acty 450
p Xylo hydroquinone in β tocopherol synthesis 445
 vit E acty 450
l Xylo 2 keto hexonic acid in vit C synthesis 303 304
l Xylose growth factor 542
 in vit C synthesis 308
 occurrence 308
 synthesis 308
l Xylosone in vit C synthesis 309
o Xylo-tocopherol 436
m Xylo tocopherol vit E acty 449
p Xylo-tocopherol 436

Y

Yeast eluate factor 197
 Yeast fermentation test for vit B₁ 131 148
 Yeast filtrate factor 253
 Yeast test for biotin 474
 for inositol 278
 for pantothenic acid 266
 for vit B₁ 211
 Yellow enzyme 154 171 172 173
 Yellow enzyme test for riboflavin 184

Z

Zinc metabolism vit B₁ relation 145
 Zwischenferment 171

